

***Durum* wheat particle size affects starch and protein digestion in vitro**

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Abstract

Purpose The term bioaccessibility refers to the proportion of a nutrient released from a complex food matrix during digestion and, therefore, becoming potentially available for absorption in the gastrointestinal tract. In the present study, we assessed the starch and protein bioaccessibility from a range of wheat endosperm products differing in particle size.

Methods Five porridge meals (size A, flour, mean particle size 0.11 mm, size B, small, mean particle size 0.38 mm, size C, semolina, mean particle size 1.01 mm, size D, medium, mean particle size 1.44 mm, size E, large, mean particle size 1.95 mm) with theoretically different postprandial glycaemic responses were subjected to oral processing in vitro, followed by simulated gastric and duodenal digestion.

Results A significant increase ($P < 0.001$) in starch degradation was observed in size A (52%) compared with size E (25%). Both sizes C and D gave less, although not significantly, digestible starch (32 and 28%, respectively). The glucose release significantly decreased as the particle size of the meal increased (92.16% detected for size A vs 47.39% for size E). In agreement with starch degradation and glucose release, size A gave the most digestible protein.

Conclusions This data provide further evidence that, by decreasing the size of wheat endosperm, starch release and glycaemic response are enhanced. We also showed that protein bioaccessibility followed a similar trend as for starch digestion. Finally, these results support the hypothesis that different degrees of starch encapsulation elicit different blood glucose responses.

Keywords Starch digestion · In vitro models · Wheat endosperm · Glycaemic response

Introduction

Starch contained in various plant structures is the major source of energy in the human diet. However, the different botanical structures (cereal grains, tubers and legumes) and other sources (buckwheat, quinoa, amaranth, sago palm and plantain) and their inherent starch type and behaviour during preparation for consumption may be expected to have an impact on behaviour in the GI tract [1, 2]. For example, cereal (monocotyledons) starch is predominantly type A and is relatively easily digested raw, whereas potato and legume (dicotyledons) starches, type B or C, are more resistant. Moreover, some starchy tissues (potato, legumes) tend to disintegrate by cell separation when cooked in the presence of water, whereas cereal grains tend to remain intact. The question therefore arises as to what effect, if any, this diversity has on the rate of digestion of the starch and subsequent absorption.

A number of studies have indicated postprandial glycaemia as a major risk factor for the development of chronic diseases, including obesity, type 2 diabetes and cardiovascular diseases [3, 4]. It is well known that the amount and structure of ingested carbohydrates are responsible for

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postprandial glucose and insulin responses [5]. Wheat is an important component in the human diet: modification of wheat-based products during food processing, together with the understanding of their behaviour in the gastrointestinal (GI) tract, has implications for functional properties. A recent study using ileostomy volunteers has revealed that the cell walls of wheat endosperm ('dietary fibre') played an important role in influencing the rate of starch amyolysis and postprandial metabolism [6]. It is hypothesised, therefore, that altering the proportion of nutrients encapsulated within the endogenous cellular (DF) structure of wheat endosperm by manipulation of particle size can affect nutrient bioaccessibility and subsequently the rates of digestion and absorption of nutrients. We refer to bioaccessibility as the proportion of a nutrient or phytochemical compound 'released' from a complex food matrix during digestion and, therefore, becoming potentially available for absorption in the gastrointestinal (GI) tract. In vitro starch amyolysis kinetic assays have been extensively used to predict glycaemic response [7]. Recently, a novel method has been developed for classifying starch digestion by modelling the amyolysis of plant foods [8].

The aim of the present study was to assess starch bioaccessibility of a range of wheat endosperm products differing in their particle size. In their in vivo study, Edwards et al. [6] compared coarsely and finely milled wheat endosperm porridge. The present in vitro study was designed to provide further insight into the mechanistic aspects of starch digestion by including a more extensive range particle size of wheat endosperm porridge with theoretically different postprandial glycaemic responses. An in vitro model of digestion consisting of a dynamic gastric model (DGM), followed by a simulated duodenal digestion phase was used. The DGM provides a realistic and predictive simulation of the physical and chemical processing of the human stomach and accurately mimics the transit time and the luminal environment within the human stomach [9, 10]. The present in vitro study will be compared with the recently published in vivo ileostomy study [6].

Materials and methods

Test meals

Five test meals were prepared from durum wheat (*Triticum durum* L.; Svevo cv.), donated from Millbo S.p.A., Trebate, Italy of various particle sizes as follows: size A (flour, mean particle size 0.11 mm), size B (small, mean particle size 0.38 mm), size C (semolina, mean particle size 1.01 mm), size D (medium, mean particle size 1.44 mm) and size E (large, mean particle size 1.95 mm). The endosperm material was prepared from de-branded

durum wheat grain using a Satake TH050 roller-mill (Satake Europe Ltd., Stockport, UK), as reported by Edwards et al. [6].

Proximate analysis of the durum wheat (protein, fat, dietary fibre by AOAC method, ash, moisture) was performed by Premier Analytical Services (High Wycombe, UK) as reported by Edwards et al. [6]. Briefly, each porridge meal made of 77 g durum wheat and 300 mL water contained: 61.1 g potentially available carbohydrate (comprised of 60.0 g starch and 0.5 g sugar), 4.5 g dietary fibre, 9.4 g protein, 1.5 g fat.

Simulated human digestion

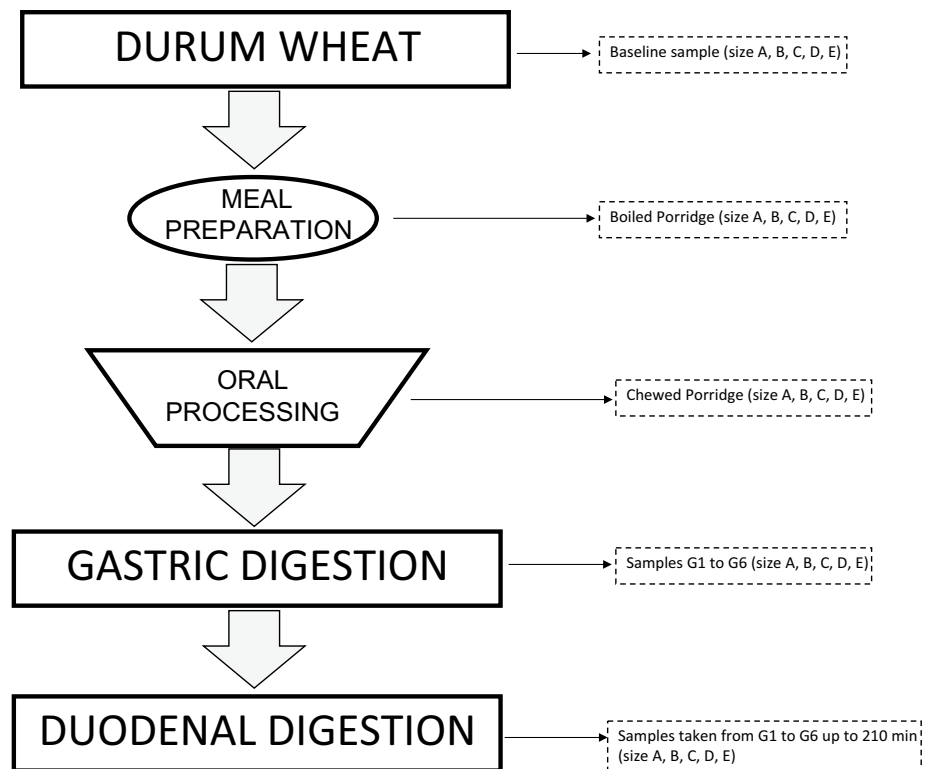
The five porridges were heated (85 °C, no boiling) in water (150 mL) while stirring vigorously for 5 min, after which cold water (150 mL) was added and the meal was heated (85 °C) for another 5 min. Next, the temperature was brought to boil for 5 min and the meal immediately transferred to a bowl, allowed to cool down and weighed. In order to avoid starch retrogradation, the time between boiling and DGM digestion was kept constant to 15 min. A flow diagram of the simulated digestion process is presented in Fig. 1.

Oral digestion

The aim of this procedure was to simulate the chewing of the five porridges (size A, B, C, D and E) in the mouth, as previously reported [10]. Simulated salivary fluid (10 mL) at pH 6.9 (0.15 M NaCl, 3 mM urea) and human salivary amylase (450 U) dissolved in simulated salivary fluid (1 mL) were added to the cooked meal and mixed for 5 min. This produced a paste consisting of equal amounts of the solid and aqueous phases as calculated by human chewing (Institute of Food Research, unpublished data). A representative subsample (2 g) was removed and added to absolute ethanol (8 mL) for subsequent analysis.

Gastric digestion

Individual porridge samples (~377 g each) subjected to oral processing were fed onto the DGM for 60 min in the presence of priming acid (20 mL), whose composition has been reported previously [9]. The priming acid is used to replicate the mean volume and concentration of acidic fluid (gastric secretions and saliva) present in the fasted stomach. The composition of the simulated gastric acid solution has been previously reported [11]. The simulated gastric enzyme solution was prepared by dissolving porcine gastric mucosa pepsin and a gastric lipase analogue from *Rhizopus oryzae* in the above described salt mixture (no acid) at a final concentration of 9000 U mL⁻¹ and

Fig. 1 Flow diagram of simulated digestion process

60 U mL⁻¹ for pepsin and lipase, respectively. A suspension of single-shelled lecithin liposomes, prepared as previously described [12], was added to the gastric enzyme solution at a final concentration of 0.127 mM. A total of six samples (G1-G6, 74 g for each meal) were ejected from the antrum of the DGM at 10-min intervals. A control digestion without addition of gastric enzymes was performed for each porridge type. Each gastric sample was weighed, its pH recorded and adjusted to 7.0 with NaOH (1 M) to inhibit gastric enzyme activity. A representative subsample (2 g) was removed at different time points (0.2, 2, 5, 10, 15, 20, 30, 40, 60, 90, 180 and 210 min) and added to ethanol (8 mL) for subsequent analysis.

Duodenal digestion

Individual gastric samples (30 g, G1 to G6) were transferred, upon ejection, to a Sterilin plastic tube for duodenal digestion with the addition of simulated bile solution (3.7 mL) and pancreatic enzyme solution (11.2 mL) and incubated at 37 °C under shaking conditions (170 rpm) for 282 min. Aliquots (2 g) were taken during duodenal incubation and added to 8 mL of ethanol for starch and glucose analysis.

Simulated bile was prepared fresh daily. It contained lecithin (6.5 mM), cholesterol (4 mM), sodium taurocholate (12.5 mM) and sodium glycodeoxycholate (12.5 mM)

in a solution containing NaCl (146.0 mM), CaCl₂ (2.6 mM) and KCl (4.8 mM).

Pancreatic enzyme solution contained NaCl (125.0 mM), CaCl₂ (0.6 mM), MgCl₂ (0.3 mM) and ZnSO₄·7H₂O (4.1 μM). Porcine pancreatic lipase (590 U mL⁻¹), porcine colipase (3.2 μg mL⁻¹), porcine trypsin (11 U mL⁻¹), bovine α-chymotrypsin (24 U mL⁻¹) and porcine α-amylase (300 U mL⁻¹) were added to the pancreatic solution.

Starch and glucose analysis

Starch analysis and glucose release were performed on the porridge samples after oral processing, gastric and gastric plus duodenal digestion using the Megazyme kit (K-TSTA 01/05).

The 80% ethanol-treated samples were centrifuged (4000g) and the supernatant used for amylolysed starch analysis (free glucose) and the pellet for starch analysis. Briefly, the pellets left after decanting the 80% EtOH were heated at 100 °C for 10 min and then dried at 40 °C under N₂. The solid was washed with 2 × 2 mL milliQ water and centrifuged at 4000 rpm for 10 min. The pellet was washed twice with 2 mL EtOH and once with acetone and air-dried for 5 or 6 days to constant weight. The pellet was broken up with a spatula or with a mortar and pestle. Triplicate 5-mg samples were dissolved in 400 μL

DMSO and vortex mixed. The tube was boiled before addition of thermostable α -amylase. After further incubation at 93 °C for 6 min, amyloglucosidase was added. Glucose released was measured at 505 nm by addition of GOPOD reagent. A regular maize starch control was included in every analytical run. From the GOPOD results the mass of starch in each pellet was calculated as starch (Glucose/110).

The 80% ethanol-treated samples were centrifuged (4000g for 2 min) and the supernatant used for amolysed starch analysis (free glucose). An aliquot (0.5 mL) from the supernatant was removed, dried at 40 °C under nitrogen and reconstituted in milli-Q water (1.0 mL). GOPOD analysis was performed on samples previously incubated with amyloglucosidase (10 μ l) to calculate the glucose (%) released.

Protein analysis

Original wheat samples and solid residues recovered after gastric plus duodenal digestion were analysed for total nitrogen by micro-Kjeldahl according to the AOAC [13], and a conversion factor of 5.70 [14] was used to calculate protein content, expressed, finally, as a percentage of dry weight.

Calculations and statistical analysis

Individual gastric samples (30 g, G1 to G6) were subjected, immediately upon ejection from the gastric model, to duodenal digestion as described above.

Aliquots (2 g) were taken during duodenal incubation over the period of 210 min and added to 8 mL of ethanol for starch and glucose analysis.

Six individual cumulative release profiles were produced. The release profiles were mathematically summed in order to obtain a single release profile which describes the total starch digestion over the overall gastric and duodenal digestion. The amount of glucose released at each time point was calculated using the experimental data of each individual duodenal digestion, and the data calculated for all the six glucose profiles were combined accordingly with the sampling time scale, to produce a total cumulative release profile. This mathematical method has been applied to all the meal particle sizes.

Statistical analysis was performed with ANOVA using SAS/SPSS and Sigmaplot software for protein and glucose, respectively. Post hoc analysis using Tukey's honestly significant difference was used to examine each pairwise difference. Means were compared using the *t*-test (paired two samples for means), and results were considered significant at $P < 0.05$. Protein data are presented as mean \pm standard error of the mean (SEM).

Results

Starch release during simulated digestion

The release of starch as a percentage of the original amount present in each porridge meal after simulated gastric plus duodenal digestion is reported in Fig. 2. As expected, a significant increase ($P < 0.001$) in starch degradation was observed in size A (52%) compared with size E (25%). Both size C and D gave less digestible starch (32 and 28%, respectively), with no significant differences compared with larger particles (size E). For all the porridge meals tested, the gastric plus duodenal digestion produced a significant increase in starch degradation over that observed in the gastric environment, where between 0.5 and 3.5% of starch was released across the different meals (results not shown). No detected starch degradation was observed after oral processing. In contrast, the physical form of the starch presented for digestion by the pancreatic α -amylase determined the extent of degradation.

Glucose release

Between 0.1 and 1% of glucose was released after simulated oral and gastric digestion. The total glucose release

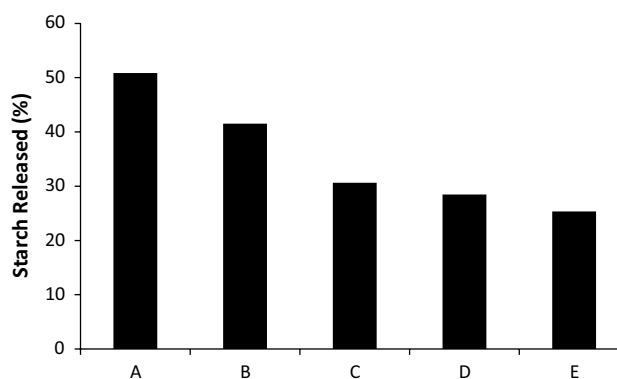


Fig. 2 Starch release as a percentage of the original amount present in each porridge meal (size A, B, C, D and E) after simulated gastric plus duodenal digestion. Values represent the average of three determinations, *standard errors* were always $<10\%$

Table 1 Glucose released from wheat porridges (size A, B, C, D and E) during simulated gastric plus duodenal digestion

Wheat	Particle size (mm)	Total release (%)
Size A	0.11	92.16 (± 4.16)
Size B	0.38	78.94 (± 1.18)
Size C	1.01	80.74 (± 2.83)
Size D	1.44	51.80 (± 4.18)
Size E	1.95	47.39 (± 1.70)

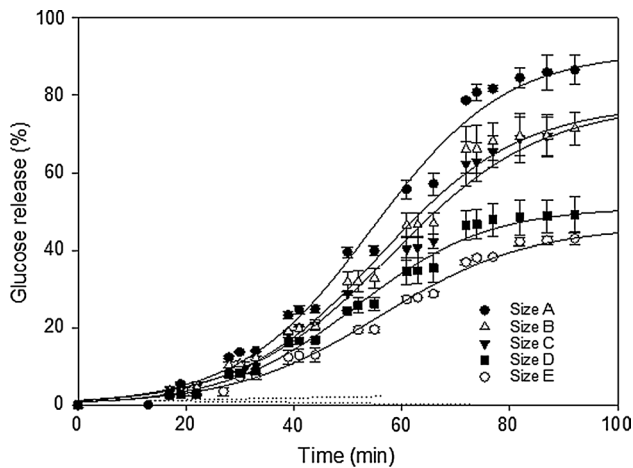


Fig. 3 Cumulative glucose release profiles during simulated gastric plus duodenal digestion as a percentage of the original amount vs time for each porridge meal (size A, B, C, D and E)

and the cumulative glucose release profiles from the five wheat porridges during simulated gastric plus duodenal digestion are reported in Table 1 and Fig. 3, respectively. These data showed that the glucose release decreased as the particle size of the meal increased. For size A, 78% of glucose was released after 72 min, reaching a plateau state after about 100 min, with a total release of 92% (Table 1; Fig. 3). Sizes B and C showed a similar release.

As shown in Table 1, the release dropped for sizes D and E, respectively, with a 46% glucose release for size D and 42% for size E. The total amount of glucose released at the end of duodenal digestion from size E porridge was decreased by about 50% compared to the reference flour.

Protein release during simulated digestion

Loss of total protein, expressed as percentage of original amount for all five porridge meals (size A, B, C, D and E) after simulated oral, gastric and gastric plus duodenal digestion, is shown in Table 2. As observed for starch degradation and glucose response, size A gave the most digestible protein and, in agreement with the starch data, the gastric plus duodenal phase was responsible for an increased release. No significant differences were observed after oral processing across all porridge sizes tested. Size C produced the highest

amount of protein released in the gastric compartment, whereas all the others samples showed an average of protein release in the range between ~14 and ~27%. We can speculate that the size of sample C may increase the functionality of gastric peptidases following a mechanical action, in which the combination of acidic conditions due to HCl and the physical interactions between particles could bring about the exposure of a major protein surface. This allows enzymes to recognise and lyse an increased amount of peptide bonds, thus resulting in an overall increase in protein release. This property depends on dimension but also on number of particles, taking into account the fixed dimension of the spaces in which it occurs. A decrease in particle size would increase the surface area:volume ratio and increase proteolysis. Larger particles would have a lower surface area:volume ratio and fewer ruptured cells, and thus make enzymolysis less efficient. In the in vitro duodenal digestion, we found almost the same release in the samples A and B with size up to 0.38 mm and a drastic decrease (over 60%) in the ones with mean particle size of 1.44–1.95 mm. This data confirm particle size has a pivotal role in protein release. In particular, samples with mean particle size up to 1.01 mm, showed high total release in protein (~60–62%), with a decrease (~33–35%) in the ones with mean particle size of 1.44–1.95 mm. The behaviour of size C samples is peculiar, with a high protein release in the gastric phase and a limited release in the duodenal compartment. In fact, although the size C particles are not big enough to limit the action of the duodenal peptidases (as for sizes D and E), this size may represent the cut-off point for good release of protein from wheat during simulated duodenal digestion. This effect could be determined by both particle size (mainly larger particles) and protection exerted by large particles on starch gelatinisation and protein denaturation.

Discussion

When starch containing particulate food is boiled, some of the surface starch becomes well dispersed in the water whereas the starch deep within the particle may only undergo limited hydration, resulting in a gel containing about 30–40% starch. When such systems are subjected to α -amylase, the initial reaction rate is high as the

Table 2 Total protein loss (%) from wheat porridges (size A, B, C, D and E) due to simulated oral, in vitro gastric and gastric plus duodenal digestion

Protein released (%)	A	B	C	D	E
Mouth	5.4 ± 1.7 ^a	9.3 ± 1.9 ^a	6.5 ± 1.5 ^a	6.9 ± 1.5 ^a	7.2 ± 1.5 ^a
Stomach	31.5 ± 3.4 ^a	26.7 ± 2.3 ^a	40.9 ± 2.6 ^b	21.2 ± 2.4 ^a	25.6 ± 1.9 ^a
Duodenum	62.6 ± 1.9 ^a	58.3 ± 2.4 ^a	59.0 ± 2.2 ^a	33.4 ± 2.2 ^b	35.1 ± 2.2 ^b
Undigested protein (%)	37.4 ± 1.9 ^a	41.6 ± 2.2 ^a	41.0 ± 2.1 ^a	66.6 ± 2.4 ^b	64.9 ± 2.2 ^b

Values are presented as mean ± SEM. Distinct letters indicate significant differences at $P < 0.05$

well-mixed fluid fraction is digested but the reaction rate declines rapidly as the process moves from the bulk phase and is confined to the surface of the particles. Additionally, as the particle is eroded from the surface, the surface area remaining shrinks exponentially (spherical model), rapidly reducing the potential rate of amylolysis.

Although the endosperm cell walls of wheat are thin (ca. 1 μm), the structure does not appear to collapse or disintegrate once the contents have been digested as seen in the micrographs of Edwards et al. [6]. This leads to an increasingly thick blanket of empty cells through which the digestive enzymes and products of hydrolysis must diffuse. The observed rapid decline in the starch hydrolysis with increasing particle size is therefore postulated to result primarily from diffusion being the limiting factor together with diminishing surface area. An additional factor may be that starch deeply embedded in larger particles is less well hydrated during cooking [15]. An understanding of the influence of these factors on glycaemia during digestion *in vivo* could indicate mechanistically how food processes may take advantage of the endogenous cellular structure to reduce the GI of otherwise high GI food.

In the present study, we have demonstrated that the physical structure of cooked wheat endosperm affected starch and protein bioaccessibility and glycaemic response *in vitro*. The importance of food structure and the role of cell wall in influencing protein availability in the gut have been previously reported using almond seeds [12]. In accordance with our previous investigation [11], all porridge meals tested produced an increase in nutrient release over and above that of the gastric phase alone. These results clearly indicated that wheat cell walls in larger particles acted as physical barrier to digestion in the upper GI tract, thus hindering starch amylolysis.

Edwards et al. [6] have recently demonstrated that cell wall encapsulation of wheat endosperm affected glycaemia, insulinemia and gut hormones in ileostomy volunteers. Blood glucose concentrations were significantly lower (<33%) after consumption of the coarse porridge (size E) than after the smooth porridge (size A). The current study was designed to improve our understanding of the effect of particle size of wheat endosperm and the mechanisms of starch bioaccessibility in the upper GI tract and its effect on postprandial glycaemia. We have shown that a particle size of 1.0 mm (surface area, size C) represented a cut-off point above which there was a decreased amylolysis.

As previously reported by other authors [16], the salivary amylase in the mouth had only minor effects on subsequent starch digestion in the small intestine by the pancreatic amylase. Although mastication and physical structure of food are thought to influence secretion rate of saliva and its amylase activity [17], our study showed no significant differences in the extent of glucose release after

oral processing across the porridge sizes. It is also believed that starch and oligosaccharides may help to protect salivary amylase against inactivation by the low pH in the gastric environment [18] although it should be noted that an inhibitory pH will not be achieved for some time after the ingestion of the meal. In the present study, we have shown glucose release within 20 s after addition of pancreatic amylase in the duodenum.

Emerging evidence has shown that slowly digestible carbohydrates which result in lower blood glucose excursions for the same starch load could help reduce the risk of cardiovascular disease and stroke [19, 20]. Silva et al. [21] have recently reported that in patients with type 2 diabetes consumption of a high glycaemic index and low fibre breakfast gave a less favourable postprandial response, with increased plasma glucose and insulin correlated with a lower satiety response. A randomised control trial to determine the glycaemic index, glycaemic load, insulinoemic index, appetite ratings and postprandial plasma concentrations of GI hormones after ingestion of five common breads consumed in Spain demonstrated that the whole-meal bread had the most favourable glycaemic response [22]. In the present study, we have shown that digestion of different porridge meals containing identical amounts of the same type of starch resulted in large variations in the extent of starch degradation. These findings may also have implications for obesity management [23]. Attenuation of changes in postprandial glycaemia has been considered a valid measure of carbohydrate quality [24].

Meynier et al. [25] have recently demonstrated that postprandial glycaemic response is affected by structural characteristics of cereal products: interaction between slowly digestible starch and rapidly digestible starch affected glycaemic response parameters of the tested cereal products. Furthermore, interaction between fat and fibres had an effect on glucose metabolism. In our study, we have shown a correlation between starch and protein bioaccessibility in the GI tract across the five porridge meals tested: we hypothesise that the physical encapsulation of starch in the large particles meals (>1 mm) dominates in controlling digestion through the limiting effect of diffusion.

We have shown that the particle sizes are also crucial for proteases action (both gastric and duodenal) and the samples with low particle sizes showed higher extent of protein degradation in both gastric and duodenal phases, with samples size C acting as a break point (cut-off at 1.01 mm) amongst all samples. Dimension above this size probably reduced the activity of proteases, thus increasing the total amount of undigested protein. Although the external surfaces are increased and available to proteases activity, wide regions are hidden to their enzymatic activity, decreasing, on the whole, the total amount of digested proteins. Overall, protein degradation, as measured by loss from the solid

food residues, was more rapid than starch. Protein release and breakdown to peptides and amino acids are crucial to meet the daily needs of organisms. Wheat is one of the major sources of plant proteins in human nutrition, but its biological value is low due to sub-optimal amounts of lysine, threonine and tryptophan.

The present study provides further evidence that, by decreasing the size of wheat endosperm particles, starch and glucose release are enhanced. We also showed that protein bioaccessibility followed a similar trend as for starch digestion. Finally, these results support the hypothesis that different degrees of starch encapsulation elicit different blood glucose responses.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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