Starch digestion kinetics and mechanisms of hydrolysing enzymes in growing pigs fed processed and native cereal based diets.

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Short version of the title: Kinetics of starch digestion in pigs.

Keywords: cereals, extrusion, *in vitro*, *in vivo*, maltodextrins.

10.1017/S0007114519000503

Abstract

This study aimed to examine *in vivo* starch digestion kinetics of starches and to unravel mechanisms of starch hydrolysing enzymes. Ninety pigs (23±2.1 kg BW) were assigned to one of nine treatments in a 3x3 factorial arrangement, with starch source (barley, maize, high amylose maize) and form (isolated, within cereal matrix, extruded) as factors. We determined starch digestion coefficients (DC), starch breakdown products, and digesta retention times in four small intestinal segments (SI1-4). Starch digestion in SI2 of pigs fed barley and maize, exceeded starch digestion of pigs fed high amylose (HA) maize by 0.20 to 0.33 DC units (P<0.01). In SI3-4, barley starches were completely digested, whereas the cereal matrix of maize hampered digestion and generated 16% resistant starch in the SI (P<0.001). Extrusion increased the DC of maize and HA maize starch throughout the SI, but not that of barley (P<0.05). Up to 25% of starch residuals in the proximal small intestine of pigs was present as glucose and soluble α (1-4) maltodextrins. The high abundance of glucose, maltose and maltotriose in the proximal SI indicates activity of brush border enzymes in the intestinal lumen, which is exceeded by α-amylase activity. Furthermore, we found that *in vivo* starch digestion exceeded our *in vitro* predictions for rapidly digested starch, which indicates that the role of the stomach on starch digestion is currently underestimated. Consequently, *in vivo* glucose release of slowly digestible starches is less gradual than expected, which challenges the predication quality of the *in vitro* assay.

Introduction

Starch is the main energy source in common pig diets. Starches in pig diets originate from various botanic origins, causing variation in digestion rate in the gastro-intestinal tract (GIT), and thus in glucose appearance kinetics in the portal circulation[(1)]. Variation in starch digestion kinetics has been demonstrated to affect pig performance. For example, pigs fed diets containing high amounts of non-digestible starch (resistant starch, RS) or slow digestible starch (SDS) had longer meal durations and inter-meal intervals[(2)], and lower energy losses by activity-related heat production[(3)], when compared with pigs fed rapidly digestible starch (RDS). Also, asynchrony between the rates of glucose- and amino acid appearance in the blood negatively affects protein utilization in restrictively fed pigs[(4)] and poultry[(5)]. In addition, variation in the presence of starch in the ileum and colon can influence the degradation of other macronutrients, notably recalcitrant fibres[(6)].

Starch is composed of two types of polysaccharides: amylose, a linear α (1-4) linked glucan, and the much larger amylopectin, an α (1-4) linked glucan that contains around 5% α (1-6) linkages, resulting in a branched molecule[7]. These two polysaccharides both form three dimensional double helices that are packed in either dense A-type crystals, or less dense B-type crystals[7]. These crystalline regions form shells which ultimately result in water-insoluble granules, which highly vary in size, shape, and porosity[8,9]. *In vitro* studies showed that these intrinsic properties of starch cause variation in starch digestion kinetics. Although many of the structural and molecular properties of starches are interrelated within a botanic source of starch, B-type crystalline structure and long amylopectin side chains generally reduce digestion rate across botanic sources[1,10,11]. Within starches of cereal origin, the number of pores and the amylopectin:amylose ratio are positively correlated with *in vitro* digestion rate[9,11]. Several *in vivo* studies confirm these *in vitro* findings, as starches with a high amylose (HA) content and B-type crystalline structure positively correlate with lower incremental plasma glucose concentrations in pigs[1,12]. Apart from intrinsic starch properties, the extrinsic cereal matrix affects digestibility. Most cereals fed to pigs are known to have two types of endosperm tissue in which starch is stored: in soft endosperm starch granules are loosely organised within the cell, whereas in hard endosperm starch is densely packed within cell walls and proteins, decreasing the degradability of endosperm tissue[13,14]. In addition, digestibility of the cereal endosperm tissue is affected by the cell wall architecture. Plant cell walls are degraded for approximately 20% when leaving the ileum of monogastrics, but the extent depends greatly on the molecular structure and composition of the cell wall[15]. Feed processing can increase starch digestion by reducing the particle size of the cereal matrix, thereby partly disrupting the cell wall matrix, and by (partly) gelatinizing the starch, for example in pelleted[16,17] or extruded diets[17].

Although *in vitro* starch digestion kinetics have been studied extensively, *in vivo* evidence is scarce, and typically focuses on ileal starch disappearance[17,18] or glucose appearance in the portal vein[19] or peripheral plasma[12]. Knowledge on starch breakdown mechanisms inside the GIT is largely based on *in vitro* studies, with a rather unknown contribution of brush border enzymes or digesta passage kinetics. Furthermore, studies on the interaction between cereal matrix, processing, and starch source towards digestion kinetics are scarce, complicating the prediction of starch digestion kinetics in a complete diet. The aim of the present study was to assess the effects of botanic source (barley, maize, high amylose maize) and starch form (isolated, in the cereal matrix or extruded) on the kinetics of starch disappearance along the GIT of pigs. Additionally, we compared the *in vivo* outcome with an

in vitro method, as described by Englyst et al[20], on starch digestion kinetics of those nine diets. The concentrations and structures of unabsorbed starch residuals in digesta were measured to increase insight into the mechanisms of starch digesting enzymes. We hypothesised that extrusion increases the rate of *in vivo* starch digestion and that an increase in amylose content decreases the rate of digestion.

Materials and methods

The experiment was conducted at research farm "Laverdonk" of Agrifirm Innovation Center (Heeswijk-Dinther, the Netherlands). All experimental procedures were approved by the Dutch Central Committee of Animal Experiments (the Netherlands) under the authorization number AVD260002016550.

Animals, Housing and Experimental design

Ninety crossbred gilts (Topigs 20 × Pietrain sire), weighing 23.1 + 2.1 kg, were assigned to one of nine treatment combinations in a 3 x 3 factorial arrangement, in four successive batches of maximum 24 pigs each. Factors were starch source (barley vs. maize vs. high amylose maize) and form (as isolated starch vs. ground cereal vs. extruded cereal). The resulting dietary treatments were abbreviated as follows: Barley starch in isolated (IB), ground (GB), and extruded (EB) form; maize starch in isolated (IM), ground (GM), and extruded (EM) form; and high amylose maize starch in isolated (IA), ground (GA), and extruded (GA) form.

In total, 96 pigs were used: 10 pigs were assigned per treatment, whereas the remaining animals served as reserve animals and were used to replace excluded animals. Seven pigs had to be excluded from the study because of feed refusals exceeding 20% of their feed allowance during the 24 hours prior to dissection. Another seven pigs were excluded due to prolonged reduction in feed intake (>4 days) and signs of an *E.Coli* infection during the experimental period. Pigs that were excluded in one of the first three batches were replaced in the sequential batch. Replacement was done such that a minimum of seven observations were realized for each dietary treatment and the number of replicates on each treatment within each batch was maintained at at least two.

The experiment consisted of an adaptation period of at least two days, during which the animals were gradually switched from a commercial grower diet (Agrifirm Feed, Apeldoorn, the Netherlands) to the experimental diets, followed by an experimental period of at least 12

days, during which the experimental diets were fed. Pigs were housed in groups of four animals per pen (0.91 m^2 per animal; 6:1 ratio of solid to slatted floor). To enable individual feeding, animals were separated using physical barriers through which they could still see, hear, smell, and touch each other. The animals remained individually housed for the duration of feeding (max 1 h per meal, two meals per day), after which they were group-housed again. Pigs always had free access to water, and pens were enriched with a toy that was changed regularly (every 2-3 days). Temperature in the barn was maintained at 25 ± 1°C. Lights were on from 6:00 to 19:00h, except for the two days before dissection (lights on from 6:00 to 22:00h) and the night prior to dissection (lights on from 2:30h onwards). Animals were fed at 2.0 × the energy requirements for maintenance (750 kJ NE per kg BW^0.60)[21], divided over two equal meals at 8:00 and 16:00h. Diets were fed as mash and mixed with water just before feeding. In the first batch, a feed:water ratio of 1:2 was applied. After the first batch, the feed:water ratio of the ground diets was altered to 1:1.5 to facilitate ingestion, whereas the feed:water ratio was maintained at 1:2 for the other treatments. During the last two days of the experimental period, the daily allowance of the pigs was equally divided over 6 meals, starting at 7:00 and applying a between-meal interval of 3 h, to reach a constant passage rate of digesta through the GIT. Just prior to dissection, a frequent feeding procedure was applied to enable the measurement of digesta passage kinetics: Each pig was fed six meals containing $1/12^{th}$ of their daily allowance each, applying a 1-hour between-meal interval. The first of the six hourly meals was fed exactly six hours before a pig was euthanized. Pigs were euthanized and dissected in an order balanced for treatment and time after onset of the frequent feeding procedure. Upon the start of the frequent feeding procedure of the first pig, extra meals ($1/12^{th}$ of daily feed allowance) were provided with two-hour intervals to the pigs whose frequent feeding procedure had not yet started, to prevent restlessness in the barns. Pigs were weighed when they entered the barns, seven days before dissection, and on the day of dissection.

Diets and processing

Nine diets, containing ~400 g of starch/kg DM, were formulated to meet or exceed the nutrient requirements of growing pigs[21] (**Table 1**). Barley grain and purified starch, isolated from the same barley grains, were obtained from Altia corporation (Koskenkorva, Finland). Maize and high amylose maize and purified starches, again isolated from the same maize grains, were obtained from Roquette (Lestern, France). Whole grains were ground by a hammer mill (3 mm sieve) and used as such, or extruded and subsequently reground by a hammer mill (3 mm sieve). Diets with isolated starch were formulated to be identical in crude

protein, fat and total dietary fibre content to diets including native or extruded grains, using soybean meal, -hulls, -protein isolate, -oil, and sugar beet pulp. Chromium oxide (Cr_2O_3) and cobalt-EDTA were included as markers in the feed at a level of 170 mg/kg (wt/wt, as fed basis).

Extrusion was performed in a co-rotating twin-screw extruder (M.P.F.50; Baker Perkins, Peterborough, UK) as described by De Vries et al.[22]. Briefly, the extruder consisted of nine heating zones and a die with two orifices (Ø 3.8 mm). Temperatures in the nine heating zones were set at 30, 40, 50, 60, 70, 80, 95, 105 and 110°C, respectively. The actual values of all heating zones were close to the set values, except the one to last zone, which was set at 105°C but reached a temperature of max 145°C. The speed of the extruder screw was fixed at 160 r.p.m. and the measured internal temperatures at the die ranged from 97 to 99°C for barley, 95 to 96°C for maize, and 95 to 97°C for high amylose maize diets. Water was added to the ground cereal directly in the extruder with a water pump at 6.8 L/h, and the measured product throughput was 55 kg dry cereals per hour. The extruded cereals were subsequently air-dried at 55°C overnight in air-forced ovens.

Digesta collection

Prior to dissection, pigs were sedated by intramuscular injection of a mixture of xylazine (2 mg/kg BW) and zolitil (4 mg/kg BW). After sedation, pigs were injected intravascular with pentobarbital (24 mg/kg BW) and exsanguinated. Immediately after exsanguination, clamps were placed between the stomach and small intestine and between the small intestine and caecum, to prevent the movement of digesta, and the organs were carefully removed. The small intestine was spread on a table and divided with clamps in four segments. The terminal 1.5 m from the small intestine (SI4) was considered to represent the ileum. The rest of the small intestine was divided in three parts with equal length (SI1, SI2 and SI3, from proximal to distal SI, respectively). All parts were dissected and their contents were collected by gently stripping. The total weight of the digesta was recorded and a representative sample was immediately frozen on dry-ice and kept at −20 °C until freeze drying. After freeze drying, samples were ground to pass a 1 mm sieve using a centrifugal mill at 12000 r.p.m. (ZM200; Retsch, Haan, Germany).

Chemical analyses

Prior to chemical analysis, feed samples were ground in the same way as digesta samples. All analysis were performed in triplicate, unless indicated otherwise. Dry matter content of

digesta was determined in singlicate by recording the weight before and after freeze drying. Dry matter content in feed was determined in duplicate according to NEN-ISO 6496[23]. Total starch content of all diet and digesta samples was determined according to AOAC Method 996.11 with the total starch assay kit from Megazyme (Wicklow, Ireland). In short, digesta and feed samples were dissolved in KOH (kit procedure c) followed by enzymatic hydrolysis of the starch (kit procedure a). The glucose concentration was determined with hexokinase-glucose-6-phosphate dehydrogenase (HK/G6P) reagent (Roche, Basel, Swiss). Samples were not washed with water or ethanol prior to analysis, thus the total starch content as measured in this study includes free glucose and soluble maltodextrins. Amylose content of starch was determined in isolated starch, according to the amylose/amylopectin procedure of Megazyme (K-AMYL 06/18). Nitrogen content of the diets was determined in duplicate according to NEN-EN-ISO 5983-2[23]. Crude fat of the diets was determined in duplicate according to NEN-ISO 6492[23]. Ash content of the diets was determined in duplicate according to NEN-ISO 5984[23]. The total dietary fibre content of the diets was calculated as total dry matter minus crude fat, nitrogen, ash, and starch[21]. Concentrations of chromium and cobalt were determined in singlicate in digesta and feed material by inductively coupled plasma optical emission spectroscopy. Chromium and cobalt were measured at a wavelength of 357.9 and 228.0 nm, respectively, as described by van Bussel et al.[24], after sample preparation according to Williams et al.[25].

The structure of unabsorbed starch residuals in the small intestine of pigs was analysed with a Scanning Electron Microscope (SEM). From each treatment, one pig was selected that had digesta mean retention times (MRT) and starch digestion coefficients (DC), in all small intestinal compartments, which were close to the average MRT and DC within that treatment. Only digesta which had more than 10% unabsorbed starch residuals (DC>0.9) could be analysed with SEM. Feed samples and fresh digesta, directly frozen after collection, were washed subsequently with hexane, twice with demi water, and finally with 96% ethanol. All washing steps were performed at room temperature, with an approximate ratio of digesta to solvent of 1:4. In between each washing step, the sample was centrifuged for 10 min at 2000 g, before the solvent was discarded. Samples were dried for 48 hours at 40 °C in an oven. Dried digesta were attached on SEM sample holders using carbon adhesive tabs (EMS, Washington, USA) and sputter coated with 15 nm tungsten (EM SCD 500, Leica, Vienna, Austria). Starch granules and granular residues were analysed with a field emission SEM (Magellan 400, FEI, Eindhoven, the Netherlands) with SE detection at 2 kV. When digesta consisted of large pieces (e.g. digesta of pigs fed ground cereals), those pieces were attached

on SEM sample holders using carbon adhesive tabs in combination with carbon adhesive (EMS). The samples were sputter coated twice, in opposite positions at angles of 45 degrees, with 15 nm tungsten.

Glucose and starch derived maltodextrins in the water soluble fractions of feed and digesta were analysed with a High Performance Anion Exchange Chromatography system with Pulsed Amperometric Detection (HPAEC-PAD). Digesta samples were pooled by intestinal segment and pig within treatment, based on weight. Diet and pooled digesta samples were boiled for 5 min (50 mg/ml) before centrifugation. Supernatant was diluted and analysed on a ICS5000 HPAEC-PAD (Dionex Corporation, Sunnyvale, CA, USA) equipped with a CarboPac PA-1 column (ID 2 mm × 250 mm) and a CarboPac PA guard column (ID 2 mm × 25 mm). The flow rate was set at 0.3 ml/min. The two mobile phases were (A) 0.1 M NaOH and (B) 1 M NaOAc in 0.1 M NaOH and the column temperature was 20°C. The elution profile was as follows: 0–37 min, 5–30.9% B; 37–50 min, 30.9–100% B; 50–55 min, 100% B; 55–55.1 min, 100–5% B; and finally column re-equilibration by 5% B from 55.1 to 65 min. The injection volume was 10 µl. Calibration curves of glucose, maltose, maltotriose, maltotetraose, maltopentaose and maltohexose were used to quantify concentrations of glucose and linear α (1-4) maltodextrins with degree of polymerization (DP) 1, 2, 3, 4, 5 and 6, respectively. Furthermore, maltohexose was used to quantify concentrations of maltodextrins with DP>6.

In vitro starch digestion kinetics were evaluated with a digestion method described by Englyst et al.[20] and Van Kempen et al.[19]. Briefly, 500 mg of starch was incubated with pepsin (P-7000) in a hydrochloric acid solution (0.05 mol/L), containing guar-gum and 50% saturated benzoic acid at pH 3 and 39°C for 30 minutes. Following, the pH was changed to 6 by adding a sodium acetate buffer (0.5 mol/L) containing porcine pancreatin (P-7545), amyloglucosidase (I4504) and invertase (A7095), and the sample was incubated at 39°C for 360 min. In contrast to the assay described by Van Kempen et al.[19], samples were incubated in a head-over-tail mixing device (8 rpm) located in an oven. Furthermore, glucose concentrations were measured in smaller aliquots in a 96 wells plate by using a glucose oxidase peroxidase assay (GOPOD, Megazyme).

Calculations and statistical analyses

In vivo digestion coefficients of starch were calculated based on the dual marker method with two indigestible markers for the insoluble (Cr_2O_3) and soluble (Co-EDTA) digesta fractions and starch concentrations in feed and digesta (equation 1)[26]. Because starch is partly

solubilized during digestion, undigested starch behaves partly as insoluble and partly as a soluble compound, which differed significantly in passage behaviour throughout the SI (unpublished data). The fraction of starch found as glucose and soluble oligomers and polymers was used to calculate DC according to Equation 1.

Equation 1: $$DC(n) = 1 - \left(\frac{[Cr_F]*(1-S)[starch_D]}{[Cr_D]*[starch_F]} + \frac{[Co_F]*(S)[starch_D]}{[Co_D]*[starch_F]} \right)$$

Where DC(n) is the digestibility coefficient of starch in the compartment n as fraction of ingested starch, [Co] is the concentration of soluble indigestible marker dosed in feed (F) or measured in digesta (D) (mg/g DM), [Cr] is the concentration of insoluble indigestible marker dosed in feed (F) or measured in digesta (D) (mg/g DM), [Starch] is the concentration of starch measured in feed (F) or digesta (D) (mg/g DM), S represents glucose and soluble starch derived maltodextrins, as fraction of the total amount of starch in digesta. In addition, digestion coefficients were calculated with Cr_2O_3 as only marker (referred to as DC_{cr}), according to the commonly used single marker method[27].

To study starch digestion kinetics, the DC was plotted against the cumulative mean retention time (CRT) of starch per segment (n) of the small intestine according to Equation 2.

Equation 2 $$CRT(n) = S * \big(MRTl(n-1) + 0.5 * MRTl(n)\big) + (1-S) * (MRTs(n-1) + 0.5 * MRTs(n))$$

Where CRT is the cumulative retention time of digesta in SI compartment n in minutes, and S is the fraction of soluble starch breakdown products as part of the total amount of starch in digesta. MRT is the mean retention time of the solid (s) or liquid (l) fraction of digesta in minutes (calculations and results will be described elsewhere). For SI1, MRT(n-1) is zero.

A modified version of the Chapman-Richards model was used to model *in vitro* digestion kinetics, as previously described by van Kempen et al.[19](Equation 3).

Equation 3 $$starch\ hydrolysis = plateau * (1 - \exp(-\frac{\frac{K}{100}}{Plateau} * 100 * time))$$

Where starch hydrolysis is expressed as % of starch in sample, plateau is the maximum amount of starch hydrolysed during digestion (as % of sample weight), which is calculated from the maximum glucose release x 0.9, and K is the rate of glucose release corrected for plateau effects (as % of starch hydrolysed to glucose per minute). Time is the incubation time (min) since start of the *in vitro* procedure. The K and plateau values of each starch sample were estimated by nonlinear regression procedures (PROC NLIN, SAS, version 9.4, SAS Institute, Cary, USA). For estimation of the plateau value, a boundary was included forcing

the estimation to be ≤1. Amounts of *in vitro* rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) were calculated based on the classification system of Englyst et al.[20].

Effects of the experimental factors on DC and DC_{cr} within each segment were tested using a general linear mixed model (PROC MIXED, SAS). Starch form (isolated starch, ground cereal, extruded cereal), starch source (barley, maize, high amylose maize), small intestinal segment (SI1, SI2, SI3, SI4) and all interactions, were included as fixed effects. Batch was included as random effect, and pig was considered as the experimental unit. Differences among starch forms within sources were considered pre-planned contrasts and were evaluated using contrast statements. Changes in DC throughout the SI within each starch source were analysed using a general linear mixed model, with segment as fixed effect. Segment within pig (subject) was modelled as R-side effect to account for repeated observations within pigs. Based on the fit statistics, a heterogeneous autoregressive covariance structure was assumed. The slice statement was used to identify effects of starch form, starch source, and their interaction within each segment, and to identify effects of segment within each starch form, starch source and source-form combination. Contrast statements were used to compare segments within starch source. Data are presented as least square (LS) means and standard deviation of the mean (S) unless stated otherwise. A retrospective power analysis was performed to validate the sample size of this study. Considering starch DC as the most important parameter, the power was evaluated using the variation in starch DC observed in this study, by calculating the critical F-value for a two-sided α level of 0.05 and for the mixed model study design[28]. A power greater than 0.95 was reached on the main effects of form, source and segment, the form x source interaction, and the source x segment interaction. For the form x segment interaction a power of 0.44 was reached and for the form x source x segment interaction a power of 0.68 was reached. Significance was assumed at $P<0.05$, while a tendency was considered when $0.05<P\leq0.1$.

Results

Effects of starch form, starch source, and small intestinal segment on starch digestion coefficients

The sum of glucose and all soluble α (1-4) maltodextrins was quantified as fraction of total unabsorbed starch residuals (**Supplemental table 1**) and used to calculate the DC of starch

(**Table 2**) In addition to the linear α (1-4) maltodextrins, other (unidentified) starch derived soluble oligosaccharides were present, making up <5% of the total peak area as measured with HPAEC, which were excluded in the calculations of starch DC. Digestion coefficients are also calculated according to the commonly used single marker method (DC_{cr}, **Supplemental table 2**).

In the absence of form x source x segment interactions (P>0.1), form x source interactions (P<0.01) are presented per segment. Due to the absence of segment x form interactions (P>0.1), segment effects on starch DC are presented within source. Overall, starch digestion increased with each following SI segment for all starch sources (0.04 to 0.32 DC units, P<0.001), except for pigs fed HA maize, where SI1 and SI2 did not differ. For all segments, a significant interaction between starch form and source was present. The average DC of starch from maize origin was similar to that of barley in SI1 and SI2, but lower in SI3 and SI4 (0.03 DC units in both segments, P<0.05). The average starch DC of pigs fed HA maize was lower than that of pigs fed barley and maize from SI2 onwards (0.20 to 0.33 DC units, P<0.01). For barley-fed pigs, starch DC did not differ among starch forms in SI1 and SI4. In SI2 the DC of starch for pigs fed GB was lower (0.16 DC units, P<0.05) and in SI3 the DC of starch for pigs fed GB was lower (0.08 to 0.09 DC units, P<0.05) than for pigs fed IB and EB. In contrast, extrusion increased the DC of starch in all SI segments of maize-fed pigs (0.12 to 0.26 DC units, P<0.05) and in all SI segments of pigs fed HA maize (0.13 to 0.47 DC units, P<0.05), compared with pigs fed ground cereals. The cereal matrix hampered starch digestion for maize fed pigs (IM vs. GM) in SI3 and SI4 (0.10 to 0.15 DC units, P<0.0001). For HA maize, the cereal matric (IA vs. GA) hampered starch digestion in SI3 (0.08 DC units, P<0.05), but not in SI4.

Glucose and maltodextrins released during starch digestion, as fraction of unabsorbed starch residuals

A typical HPAEC elution pattern of the soluble fraction of starch residuals in small intestinal digesta (SI1-SI4) of pigs fed ground maize (**Supplemental figure 1**) illustrates the presence of mainly glucose and linear α (1-4) maltodextrins. The fraction of unabsorbed starch residuals (1-DC) was divided into glucose, individual α (1-4) gluco-oligosaccharides (up to DP6), soluble gluco-polysaccharides (>DP6) and insoluble starch (**Figure 1**). The sum of glucose and all soluble maltodextrins is referred to as soluble unabsorbed starch residuals. Expressed as a percentage of total unabsorbed starch residuals, these soluble residuals averaged 25% in SI1 and 18% in SI2 of barley-fed pigs, whereas this was only 4% in SI3 and

2% in SI4. For pigs fed maize based diet, a similar pattern was observed as 17, 13, 4 and 1% of total unabsorbed starch residuals was recovered as soluble starch residuals in SI1 to SI4, respectively. For HA maize-fed pigs, soluble starch residuals made up 10, 11, 9 and 7% of the total unabsorbed starch residuals from SI1 to SI4, respectively. For all treatments, concentrations of glucose, maltose and maltotriose were numerically highest of all individually identified molecules. For barley-fed pigs, the sum of glucose, maltose and maltotriose averaged 32% of the soluble unabsorbed starch residuals across all SI segments, whereas this was 24% for maize-fed pigs and 12% for pigs fed HA maize. In the first SI segment of pigs fed extruded cereals, the sum of glucose, maltose and maltotriose constituted 33% of the soluble unabsorbed starch residuals, which was 21% for pigs fed isolated starch and 13% for pigs fed ground cereals. In SI2-4, this concentration averaged 32% for pigs fed isolated starch, 19% for pigs fed ground cereals and 17% for pigs fed extruded cereals.

Visual inspection of undigested starch

Prior to ingestion, starch consisted largely of undamaged starch granules, both at individual granule level (**Figure 2**, 5000x magnified) and within the ground cereal matrix (**Figure 3**, 1000x magnified). Furthermore, starch in all ground cereals was mainly present inside a protein and cell wall matrix, which was damaged upon extrusion (**Figure 3**). Individual starch granules of diets containing isolated starches showed signs of digestion in all parts of the SI, although digestion appeared more extensive for barley and maize starch granules compared with high amylose maize starch (**Figure 2**). Digestion of starch fed as ground cereals was hampered by the protein and cell wall matrix, which remained for a part intact throughout the small intestine (**Figure 4** and **Supplemental figure 2**).

In vitro starch digestion kinetics

For all starch sources, the rate of *in vitro* starch digestion was measured and was found to be higher for extruded diets compared with diets containing isolated starch and ground cereals (**Table 3**). Furthermore, each high amylose maize starch was digested slower *in vitro* than barley and maize starches of the same form. Extrusion resulted in a substantial increase in RDS, which was around 20% higher in barley and maize starch compared with high amylose maize starch. Consequently, extruded cereals contained low amounts of SDS and little (HA maize) or no RS (barley and maize). Ground barley and maize were digested slower than isolated barley and maize starch, resulting in higher levels of RDS for IB versus GB and IM versus GM. In contrast, isolated HA maize starch and ground HA maize were digested at a

similar rate, resulting in similar levels of RDS, which were much lower than RDS levels of IB, IM, GB and GM. Consequently, IA and GA contained considerable higher levels of RS, but not SDS, than all other diets.

Discussion

The aim of this study was to assess the effects of variation in botanic starch source and processing form on the kinetics of starch disappearance along the GIT of pigs and to relate this to the *in vitro* predicted digestion kinetics. In addition, the structure of starch residuals that remained unabsorbed in the small intestine were analysed to obtain more information on the digestive mechanisms of starch hydrolysing enzymes.

Influence of intrinsic starch properties vs. the cereal matrix

An in-depth analysis of the intrinsic properties of maize, barley and high amylose maize starch and their relation to *in vitro* digestion kinetics is presented elsewhere[11]. Briefly, maize and barley starch used in this study have comparable intrinsic properties, whereas high amylose maize has a higher amylose content (55%) compared with barley and maize starch (20%). Furthermore, HA maize starch has a different type and amount of crystalline structure, less pores, and a different amylopectin structure. These intrinsic properties of HA maize hampered ileal digestibility, illustrated by the lower ileal starch digestibility of IA (66%) compared with IM (99%), which confirms results of *in vivo* studies[29-31].

Presence of the cereal matrix hampered ileal starch digestion for maize but not for barley and HA maize. This is illustrated by a reduced ileal starch DC for pigs fed ground vs. isolated and maize, whereas this difference is absent for barley and HA maize. Results of *in vivo* studies with pigs have indicated that a reduction of the particle size, thus an increased damage of protein matrix and cell walls, increased ileal starch digestibility of both barley, from 0.92 to 0.96 units[18], and maize, from 0.89 to 0.97 units[32]. Additionally, a reduction of the particle size of maize increased starch DC in the duodenum and jejunum of pigs[33].

In this research, neither cereal endosperm nor cell wall structures were examined. Analysis with SEM revealed undigested protein residues covering starch granules in the distal SI parts of pigs fed ground maize (**Figure 4**). This indicates the presence of substantial fractions of hard endosperm, which is typically richer in indigestible proteins[14]. In contrast, only loosely packed starch granules were identified for barley (**Figure 4**), suggesting the presence of mainly soft endosperm that allows for a more rapid starch digestion[34]. SEM analysis also

revealed large fractions of starch granules entrapped within intact cell wall material in SI4 of maize-fed pigs (**Supplemental figure 2**), which was not observed for barley-fed pigs. Based on previous research, it is suggested that this is caused by larger fractions of soluble fibres, which are generally more abundant in barley than in maize, and more easily degraded by monogastrics[15,35].

In summary, both the endosperm cell wall and protein structure seem to contribute to a higher RS fraction in ground maize compared with ground barley. In ground high amylose maize, the cereal matrix has likely a similar effect as seen in ground maize, as substantial fractions of hard endosperm were observed in HA maize. However, intrinsic properties of high amylose maize seem to hamper digestion more than its cereal matrix, because the DC of isolated high amylose maize did not exceed the DC of ground high amylose maize in any of the SI parts.

The effect of extrusion on in vivo *starch digestion*

Extrusion increased the ileal digestibility of maize and high amylose maize starch with 0.15 and 0.19 units to 0.98 and 0.79 units, respectively. This is more than expected based on previous research with maize-fed pigs, where a modest increase in ileal digestibility of 0.02 units was identified[17]. However, the starch DC measured for native ground maize used in the current study (0.86) was lower than in the previous study (0.98)[17]. Ileal starch digestibility of ground barley was nearly complete in our study, leaving no room for an increase by extrusion, which was observed in previous work[36]. For HA maize, starch DC in the proximal SI was increased by extrusion, whereas the DC remained almost similar to that of SI1 in subsequent SI segments. As visualized by SEM, the effect of extrusion on high amylose maize appeared smaller than observed for maize and barley (**Figure 3**). Indeed, the molecular properties of high amylose starches lead to a higher gelatinization temperature of starch, causing similar processing conditions to result in a lower degree of gelatinization[37-39].

Mechanisms of starch hydrolysing enzymes in the SI

A substantial part of the unabsorbed starch residuals in the small intestine was present as soluble oligomers (**Figure 1**), especially for pigs fed barley and maize (on average 63 and 42% across starch forms, respectively). Our novel findings indicate that the soluble oligomer fraction in SI1 and SI2 consisted mostly of molecules with DP≤3. Maltose and maltotriose are typical end-products of pancreatic α-amylase[40], whereas glucose is the end-product of brush border enzyme activity[41]. The presence of glucose in the proximal SI suggests a delay in the absorption of glucose that is produced by brush border enzymes, which was observed before

for pigs fed native maize starch[42]. In addition, the high concentrations of glucose in digesta indicates activity of brush border enzymes, which are not bound to the gut wall. This corresponds well with results of recent experiments, which indicated that a proportion of brush border enzymes is actively budded off as brush border membrane vesicle[43] and that the enzymes might transit and diffuse to all parts of the intestinal lumen[44]. The presence of maltose and maltotriose reveals that the rate of starch hydrolysis by α-amylase exceeds the rate of maltose and maltotriose degradation by brush border enzymes.

In digesta of pigs fed isolated barley or maize starches, granular starch residues in SI1 and SI2 showed severe signs of digestion (**Figure 2**), whereas little to no granular residues were left in SI3 and SI4. Digesta of pigs fed IA contained granular starch residuals in all SI compartments. Granules remaining in SI4 showed barely signs of digestion, indicating that granules are either fully digested, or left untouched. This heterogeneous digestion of high amylose starch has been observed previously *in vitro*, where indeed most residual granules from high amylose maize starch were largely intact[45,46].

Comparing in vivo *starch digestion kinetics with an* in vitro *assay*

The rate of *in vitro* starch digestion measured in this study was higher for extruded cereals compared with ground cereals, whereas that of ground cereals was higher than that of isolated starches. This confirms results of *in vitro* studies, which showed that the presence of a cereal matrix slows starch digestion[34,47] and that extrusion increases starch digestion rates[36]. In addition, every form of high amylose maize starch analysed in this study was digested slower than maize starch of the same form. This is also in agreement with *in vitro* results demonstrating a negative correlation between digestion rates and an increased amylose content, B-type of crystalline structure, and long amylopectin side-chains[19,30,48,49].

In vitro and *in vivo* hydrolysis rates were visually compared (**Figure 5**), by plotting *in vivo* starch hydrolysis in all segments of all pigs on a single treatment, against the cumulative intestinal retention time (CRT, **Supplemental table 3**). For this plot, maltodextrins with DP≤3 were assumed to be end products of α-amylase hydrolysis[40]. In the same figure, *in vitro* starch hydrolysis was plotted against the incubation time. For extruded starches, the initial rate of starch digestion, in SI1 and SI2, compares well between *in vitro* and *in vivo* data. For isolated and ground starch sources, however, the *in vitro* assay underestimates the initial rate of starch digestion. The extent of *in vivo* starch digestion in SI1 measured in this study (on average 35% for all diets) is close to that in the duodenal and initial jejunum of growing pigs fed ground maize (on average 45%)[33]. Additionally, it corresponds well with

the extend of starch digested in the first third of the SI of growing pigs fed ground oats (on average 57%)[50]. The difference between *in vitro* and *in vivo* starch digestion becomes smaller towards the distal SI for barley and corn starches in isolated or ground form, whether it remains rather constant for IA and GA.

The similarity between our *in vivo* data and results of previous studies[33,50], emphasizes that the *in vitro* method systematically underestimates the initial *in vivo* rate of starch digestion. This contributes to the on-going debate on the predictability of *in vivo* data by *in vitro* assays[51,52]. Amongst others, the absence of brush border enzymes in the *in vitro* assay may result in an underestimation of *in vivo* starch digestion[53,54]. Alternatively, digestion processes initiated in the stomach may partly explain the rapid initial starch hydrolysis *in vivo*. This includes the possibility of starch hydrolysis in the stomach, but also alterations of the digesta matrix, inadequately simulated *in vitro*. Finally, errors in the measurement of *in vivo* digesta passage kinetics may also contribute to differences between *in vivo* and *in vitro* results.

All starch that is digested *in vitro* in 120 min, but not in 20 min, can be considered SDS according to Englyst' classification[20]. This fraction corresponds to a gradual and prolonged glucose release *in vivo*, leading to an extended glycaemic index[55]. Barley and maize diets, containing starch in isolated or ground form, were high in SDS according to *in vitro* digestion, but did not release a relevant amount of glucose in the distal part of the small intestine. IA and GA contained 33% *in vitro* digestible starch, of which 20% was characterized as RDS and 80% as SDS. In contrast, IA and GA were *in vivo* digested for 65 and 68%, respectively, of which half disappeared from the SI within the first 10 minutes. The fraction of intact starch barely differed between SI3 and SI4 (**Figure 1**), which indicates that starch escaping initial hydrolysis *in vitro* does not necessarily leads to an increase in starch hydrolysis in more distal parts of the small intestine. Consequently, the *in vivo* glucose release, and thus glucose absorption, is less gradual than expected based on *in vitro* analysis.

Conclusions

Starch digestion for barley and maize is primarily determined by the cereal matrix, whereas digestion of HA maize is limited by intrinsic starch properties. The presence of soluble maltodextrins in SI digesta illustrates that brush border enzyme activities are rate limiting in rapidly digestible starches, but variation in starch digestion kinetics, caused by the feed matrix, is not adequately predicted by current *in vitro* methods. The underestimation of initial starch digestion *in vitro* indicates that the role of the stomach on starch digestion is currently

underestimated. The current results indicate that glucose release from slowly digestible starch is less gradual than predicted from *in vitro* analysis.

Acknowledgements

Financial support

This project is jointly financed by the Topsector Agri&Food and Agrifirm as coordinated by the Dutch Carbohydrate Competence Center (CCC-ABC; www.cccresearch.nl).

Conflict of interest

All authors declare that they have no conflicts of interest.

Authorship

BMJM, HAS, EMAMB, and WJJG designed the experiment. BMJM and TF conducted research. BM, SV, and WJJG performed statistical analysis. BMJM wrote the manuscript. SV, HAS, EMAMB, and WJJG revised the manuscript. All authors have read and approved the final manuscript.

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Tables

Table 1. Ingredient and nutrient composition of diets containing barley, maize, or high amylose maize starch, included as isolated powder, ground cereal, or extruded cereal[1].

Inclusion level (as is basis)	IB	GB	EB	IC	GC	EC	IA	GA	EA
Barley starch,[2] *g/kg*	441.0								
Ground barley,[2] *g/kg*		800.0							
Extruded barley,[2] *g/kg*			800.0						
Maize starch,[3] *g/kg*				441.0					
Ground maize,[3] *g/kg*					668.9				
Extruded maize,[3] *g/kg*						668.9			
High amylose maize starch,[3] *g/kg*							441.0		
Ground high amylose maize,[3] *g/kg*								745.3	
Extruded high amylose maize,[3] *g/kg*									745.3
Soybean meal, *g/kg*	110.0			110.0			110.0		
Sugar beet pulp, *g/kg*	50.8			50.8			50.8		
Soybean hulls, *g/kg*	200.0			200.0	109.0	109.0	200.0	56.7	56.7
Soybean protein isolate,[4] *g/kg*	92.0	105.1	105.1	92.0	132.7	132.7	92.0	109.6	109.6
Soy oil, *g/kg*	54.8	36.9	36.9	54.8	30.7	30.7	54.8	21.6	21.6
Dicalciumphosphate, *g/kg*	27.4	22.3	22.3	27.4	27.6	27.6	27.4	27.9	27.9
Mineral and vitamin premix,[5] *g/kg*	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Salt (NaCl), *g/kg*	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
L-lysine HCl, *g/kg*	4.6	5.7	5.7	4.6	5.0	5.0	4.6	7.3	7.3
$NaHCO_3$, *g/kg*	4.0	4.9	4.9	4.0	4.2	4.2	4.0	5.2	5.2
$KHCO_3$, *g/kg*	2.8	8.0	8.0	2.8	8.4	8.4	2.8	9.8	9.8
DL-Methionine, *g/kg*	2.9	1.5	1.5	2.9	1.5	1.5	2.9	2.2	2.2
L-threonine, *g/kg*	1.5	1.1	1.1	1.5	0.8	0.8	1.5	1.8	1.8
$CaCO_3$, *g/kg*	0.3	6.5	6.5	0.3	3.3	3.3	0.3	4.4	4.4
L-tryptophan, *g/kg*								0.3	0.3
Cr_2O_3 , *mg/kg*	170.0	170.0	170.0	170.0	170.0	170.0	170.0	170.0	170.0
Co-EDTA, *mg/kg*	170.0	170.0	170.0	170.0	170.0	170.0	170.0	170.0	170.0
Analysed nutrient composition (DM basis)									
Starch, *g/kg*	423	444	470	423	472	482	401	467	474
Amylose, as % of starch	20	20	20	20	20	20	55	55	55
Protein, *g/kg*	189	190	192	191	200	189	194	199	192
Fat, *g/kg*	66	56	48	65	64	45	66	60	42
Ash, *g/kg*	63	64	63	65	65	62	64	64	62
Moisture, *g/kg as is*	102	105	59	105	111	65	107	114	67
Energy and apparent ileal digestibility levels of phosphorus and amino acids[6]									
Net Energy, *MJ/kg DM*	10.5	10.1	10.1	10.5	10.2	10.2	10.5	10.6	10.6
Phosphorus, *g/kg DM*	7.2	8.2	8.2	7.2	8.5	8.5	7.2	8.5	8.5
Lysine, *g/NE*	10.8	10.8	10.8	10.8	10.8	10.8	10.8	10.8	10.8
Methionine + cysteine, *g/NE*	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Threonine, *g/NE*	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Tryptophan, *g/NE*	2.0	2.2	2.2	2.0	2.1	2.1	2.0	2.0	2.0

[1] Diets are abbreviated as follows: Barley starch in isolated (IB), ground (GB), and extruded (EB) form; maize starch in isolated (IM), ground (GM), and extruded (EM) form; and high amylose maize starch in isolated (IA), ground (GA), and extruded (GA) form.
[2] Altia corporation, Koskenkorva
[3] Roquette, Lestern, France
[4] Unisol NRG IP Non-GMO, Vitablend, Wolvega, The Netherlands
[5] Provided per kg of diet: Vitamin A (retinyl acetate), 10,000 IU; vitamin D_3 (cholecalciferol), 2,000 IU; vitamin E (DL-α-tocopherol), 40 mg; vitamin K_3 (menadione), 1.5 mg; vitamin B_1 (thiamin), 1.0 mg; vitamin B_2 (riboflavin), 3 mg; vitamin B_6 (pyridoxine-HCl), 1,5 mg; vitamin B_{12} (cyanocobalamin), 20 µg; niacin, 30 mg; D-pantothenic acid, 15 mg; choline chloride, 150 mg; folic acid, 0.4 mg; biotin, 0.05 mg; Fe, 100 mg, as $FeSO_4 \cdot H_2O$; Cu, 20 mg, as $CuSO_4 \cdot 5H_2O$; Mn, 30 mg, as MnO; Zn, 70 mg, as $ZnSO_4 \cdot H_2O$; I, 1 mg, as KI; Se, 0.25 mg, as Na_2SeO_3
[6] Calculated based on data from Centraal Veevoeder Bureau[(21)]

Table 2. Digestion coefficients (DC) of starch in digesta recovered from four consecutive parts of the small intestine of pigs fed diets containing barley, maize, or high amylose maize starch, included as isolated powder, ground cereal, or extruded cereal.[1,2,3]

DC	Experimental diets[4]									S	p – value[5]			Effect[6]	
	Barley			Maize			High amylose maize								
	I	G	E	I	G	E	I	G	E		Form	Source	F*S	Form	Source
Max obs[7]	10	10	9	10	10	9	7	7	10						
SI1	0.40	0.28	0.47	0.34^{fg}	0.19^{g}	0.45^{f}	0.20^{l}	0.16^{l}	0.63^{k}	0.22	**<0.0001**	0.592	**0.0005**	E>I=G	
SI2	0.68^{a}	0.52^{b}	0.64^{ab}	0.57^{g}	0.60^{g}	0.78^{f}	0.29^{l}	0.35^{l}	0.59^{k}	0.18	**0.0009**	**<0.0001**	**<0.0001**	E>I=G	B=M>A
SI3	0.96^{a}	0.87^{b}	0.95^{a}	0.92^{f}	0.82^{g}	0.94^{f}	0.50^{m}	0.58^{l}	0.71^{k}	0.06	**<0.0001**	**<0.0001**	**<0.0001**	E>I>G	B>M>A
SI4	0.99	0.95	0.97	0.99^{f}	0.84^{g}	0.98^{f}	0.55^{l}	0.60^{l}	0.79^{k}	0.07	**<0.0001**	**<0.0001**	**<0.0001**	E>I>G	B>M>A
p-value[8]	**<0.0001**			**<0.0001**			**<0.0001**								
Effect[9]	SI1<SI2<SI3<SI4			SI1<SI2<SI3<SI4			SI1=SI2<SI3<SI4								

[1] Presented values are estimated LSMeans and standard deviation (S).
[2] DC values are calculated using the dual-marker method[(26)].
[3] SI4 is the terminal 1.5 m of the small intestine, whereas the rest of the small intestine is divided in three parts with equal length (SI1, SI2 and SI3, from proximal to distal SI, respectively).
[4] Starch forms and sources are abbreviated as follows: isolated (I), ground (G), and extruded (E) form, originating from barley (B), maize (M), and high amylose maize (A).
[5] Model established p-values for fixed effects of starch form (isolated, ground, or extruded), source (barley, maize, or high amylose maize), and the interaction between form (F) and source (S), within segment. When an interaction between form and source was identified, superscripts [a,b] indicate differences among starch forms within all diets of barley origin ($p<0.05$). Superscripts [f,g] indicate differences among starch forms within all diets of maize origin ($p<0.05$). Superscripts [k,l,m] indicate differences among starch forms within all diets of high amylose maize origin ($p<0.05$).
[6] When a form or source effect is present ($P<0.05$), ">" indicates that the DC of a form/source is larger than others, whereas "=" indicates that there is no difference in DC.
[7] The maximum number of replicate observations equals the amount of animals per treatment. In some segments, not enough digesta was present to allow chemical analysis, causing one missing observation in SI1 of GB, SI1 of EA, SI4 of IB, and SI4 of GM, and two missing observations in SI1 of EM.
[8] Model established p-values for fixed effects of segment, analysed per source.
[9] When a segment effect is present ($P<0.05$), "<" indicates that the DC of a segment is smaller than others, whereas "=" indicates that there is no difference in DC.

Table 3. *In vitro* digestion rate, plateau level and calculated amounts of rapidly digestible starch (RDS)[1], slowly digestible starch (SDS), and resistant starch (RS) of diets containing barley, maize, or high amylose maize starch, included as isolated powder, ground cereal, or extruded cereal.

	Experimental diets								
	Barley			Maize			High amylose maize		
	Isolated	Ground	Extruded	Isolated	Ground	Extruded	Isolated	Ground	Extruded
Rate (%/min)	4.0	1.8	15.1	2.3	1.8	13.0	0.4	0.4	7.1
Plateau (%)	100	99	100	98	99	100	67	75	89
RDS (%)	54.6	30.6	94.3	36.4	29.9	91.3	7.2	7.2	70.4
SDS (%)	44.2	57.1	5.4	55.2	57.5	8.7	25.8	26.0	18.4
RS (%)	1.2	12.3	0.2	8.5	12.6	0.0	67.0	66.7	11.2

[1] Calculated based on the classification system of Englyst et al.[20].

Figure titles

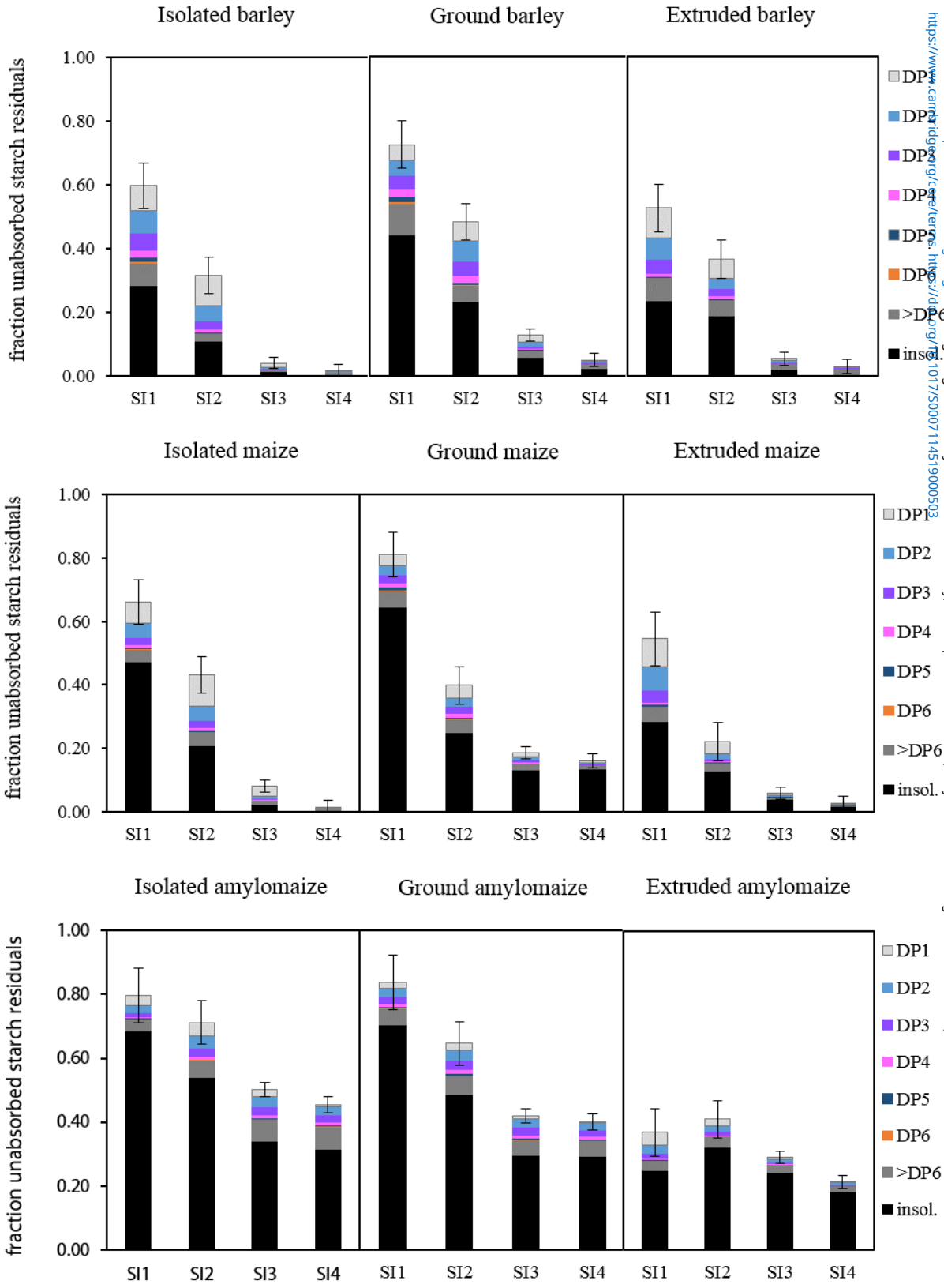
Figure 1. Fraction unabsorbed starch residuals, calculated as 1 - digestion coefficient (DC) in digesta recovered from 4 parts of the small intestine of pigs fed barley, maize or high amylose (HA) maize based diets which included starch as isolated powder, ground cereal or extruded cereal. Undigested starch is divided into soluble oligomers, quantified per individual oligomer up to degree of polymerization (DP) 6, and insoluble starch. The error bars represent the standard error of the estimated mean DC.

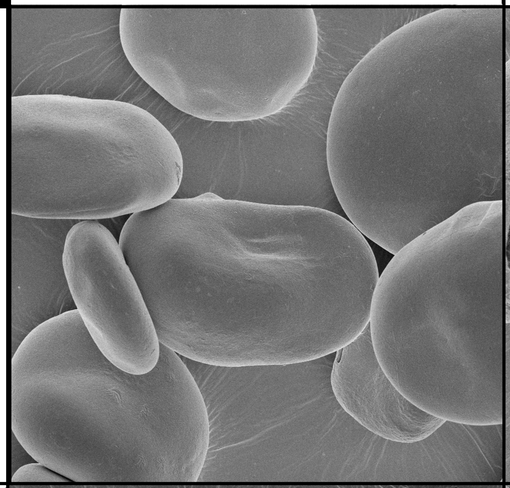
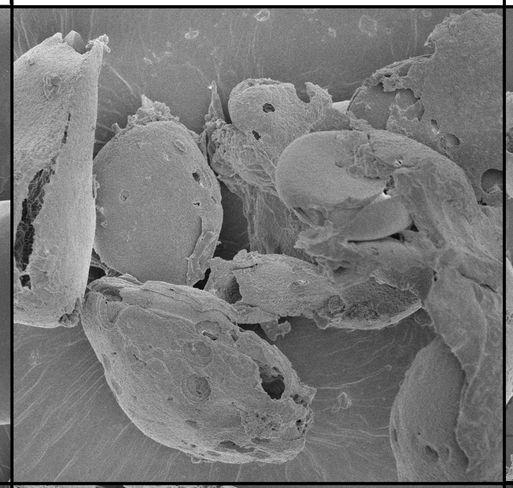
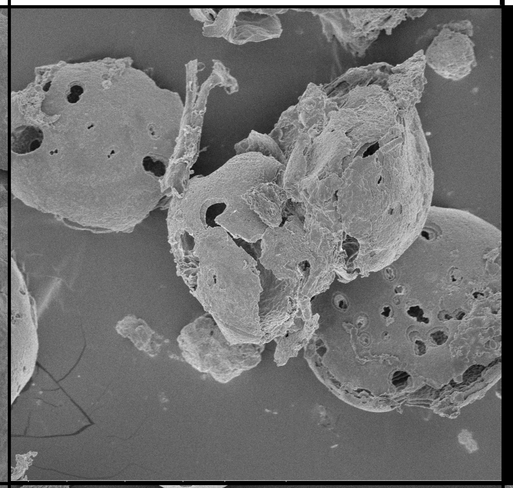
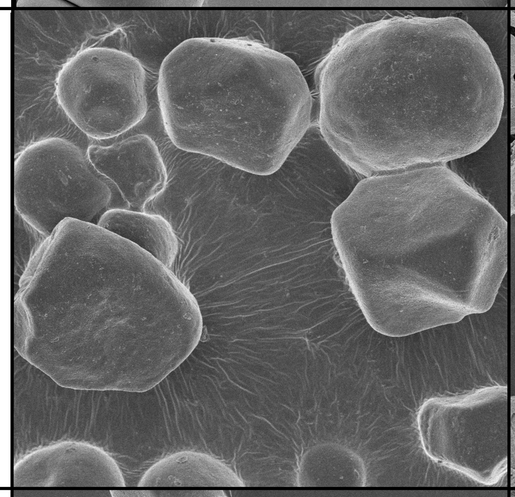
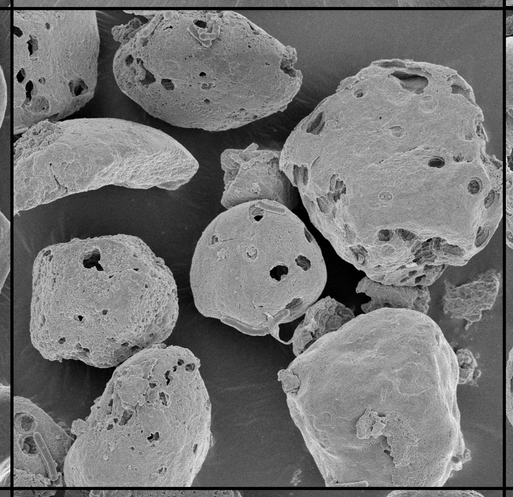
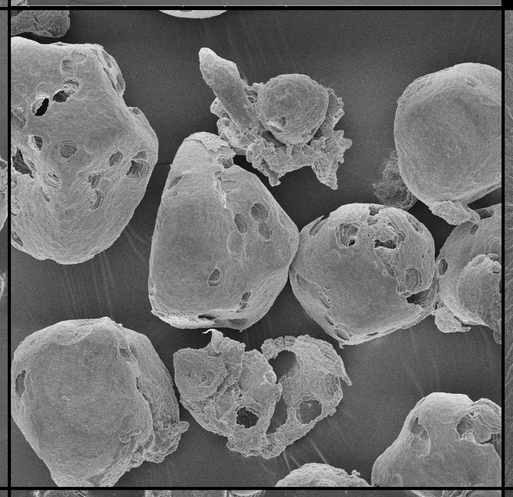
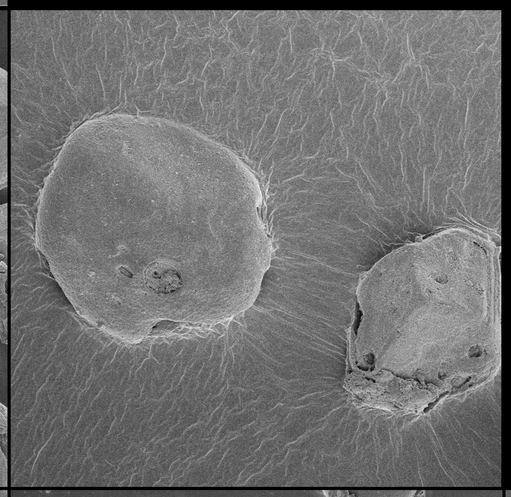
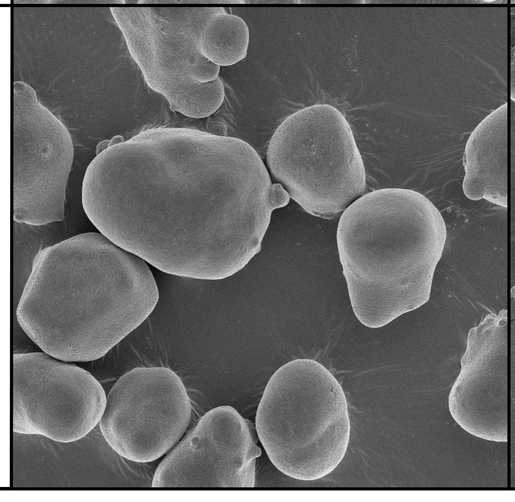
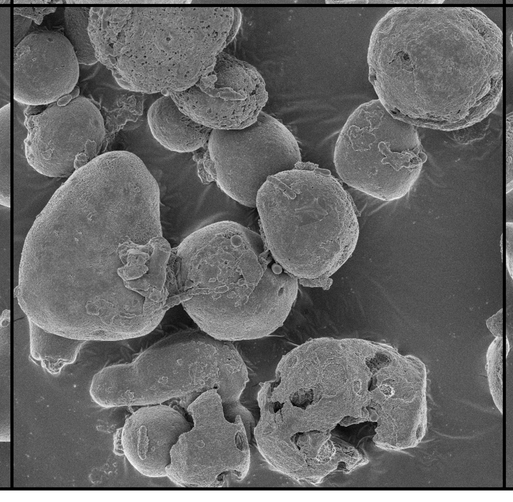
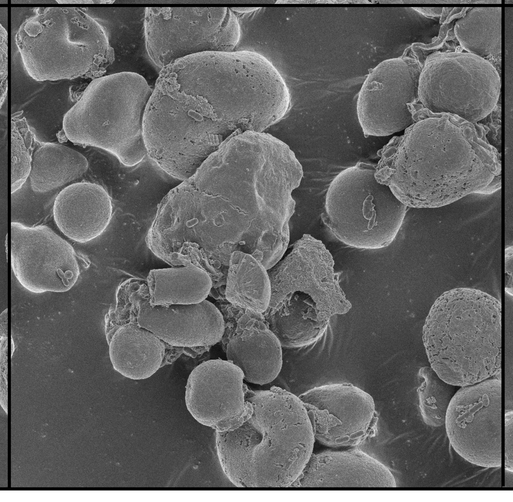
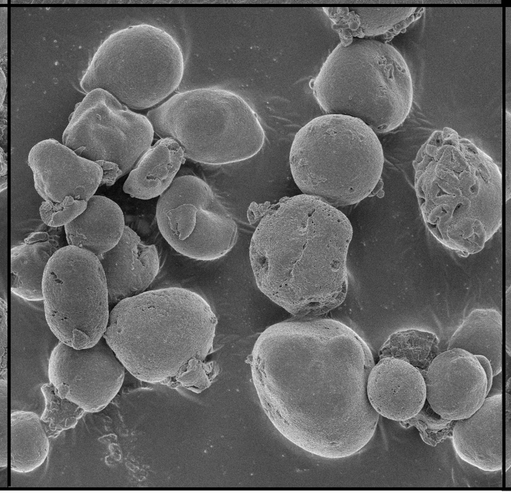
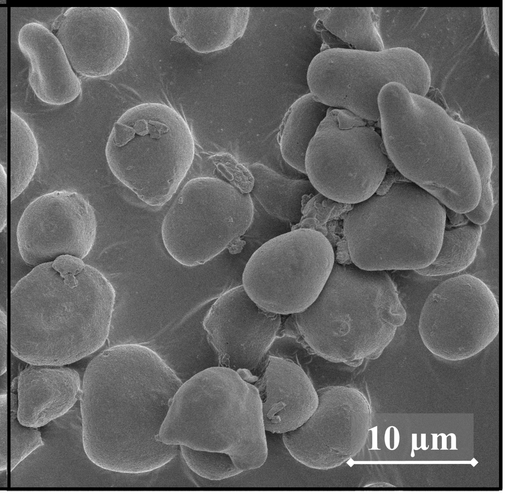
Figure 2. Typical SEM images of starch granules in digesta of pigs fed diets containing isolated starches from barley, maize, or high amylose maize origin, in diets and 4 segments of the small intestine, 5000x magnified. N.D. is used to indicate that not enough insoluble starch residues were present in those SI compartments to enable SEM analysis.

Figure 3. SEM images of diets containing barley in ground (A) and extruded form (B), maize in ground (C) and extruded form (D), and high amylose maize in ground (E) and extruded form (F), 1000x magnified.

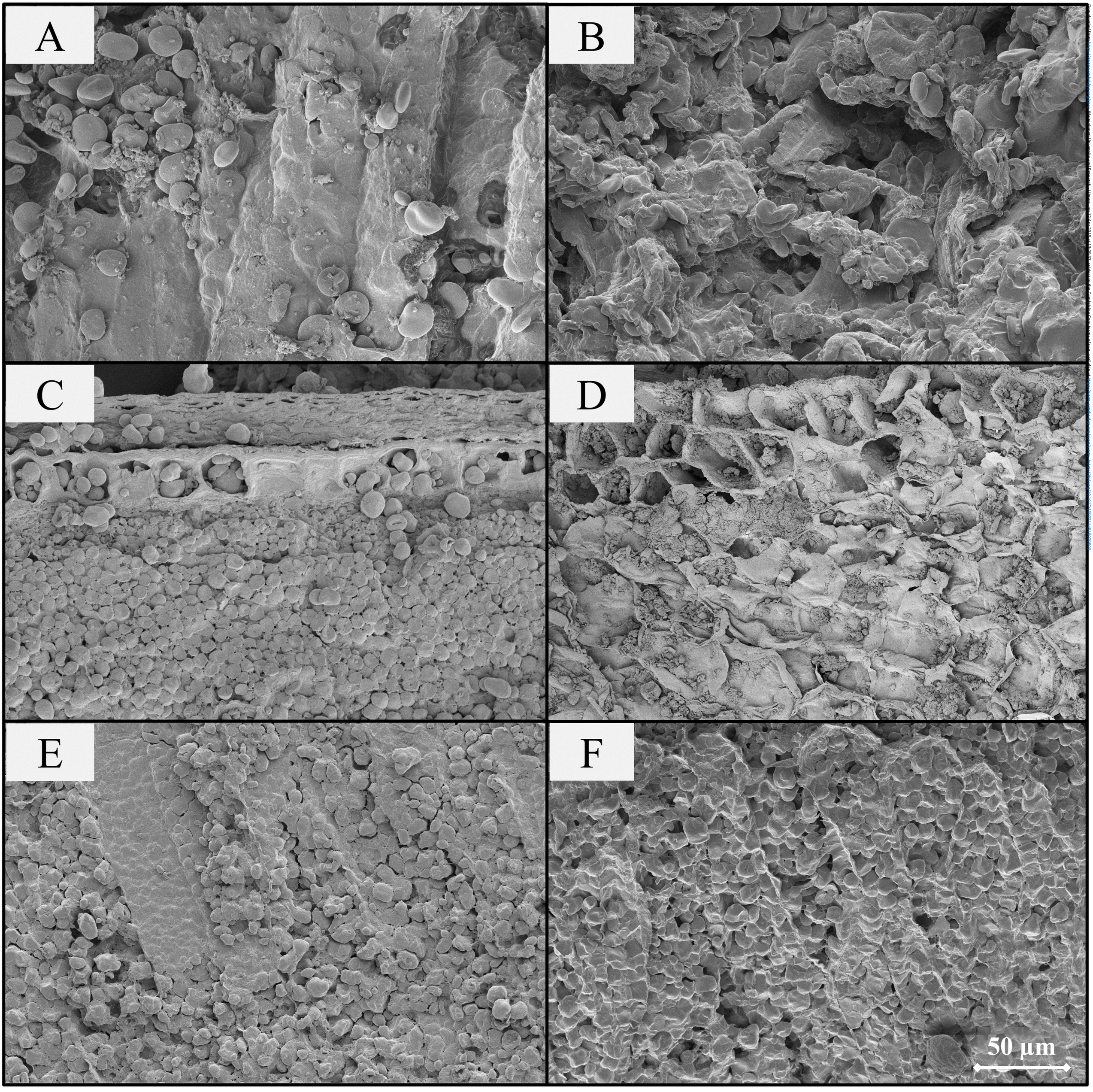
Figure 4. SEM image of digesta recovered from SI4 of a pig fed ground maize (A) and of a pig fed ground barley (B), 5000x magnified.

Figure 5. Digestion coefficients (DC) of starch measured in digesta recovered from part 1(♦), 2 (□), 3(○) and 4(Δ) of the small intestine of individual pigs fed barley, maize or high amylose maize based diets which included starch as isolated powder, ground cereal or extruded cereal, plotted against the CRT. In each graph, *in vitro* starch hydrolysis is plotted against incubation time. Symbols (●) indicate the average of in triplicate measured values; lines represent the first-order kinetic model fitted to the data points. Error bars represent the standard deviation of *in vitro* measured starch digestion.



	Diet	Small intestine 1	Small intestine 2	Small intestine 3	Small intestine 4
Isolated barley				N.D.	N.D.
Isolated maize					N.D.
Isolated HA maize					

10 µm


A
B
C
D
E
F
50 µm