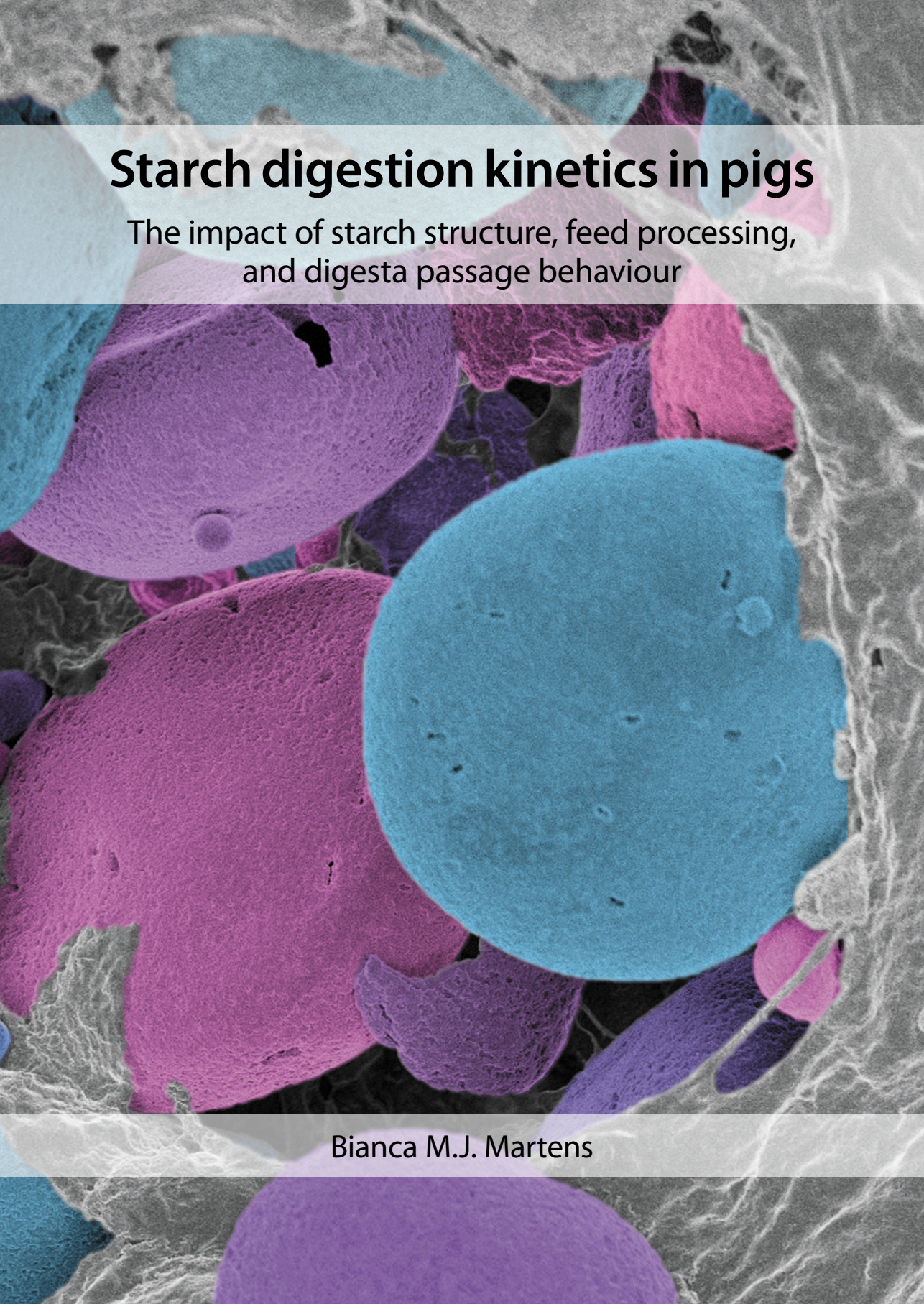

Starch digestion kinetics in pigs

The impact of starch structure, feed processing, and digesta passage behaviour

Bianca M.J. Martens

Propositions

1. Slowly digestible starch does not exist in pigs.
   (this thesis)

2. It is useless to discuss starch digestion kinetics without including the contribution of gastric bacteria.
   (this thesis)

3. Misrepresentation of risks by mass media causes suboptimal prioritization of public resources. (Plos one. 2008; 3(10): e3352)

4. Our copycat behaviour initiated "the age of humans". (Science. 2007; 317(5843) pp. 1360)

5. Justice would benefit if an algorithm was to determine the sentence for convicts.

6. Animal welfare conveniently lacks focus on pet animals.

Propositions belonging to the thesis, entitled
Starch digestion kinetics in pigs
The impact of starch structure, feed processing,
and digesta passage behaviour

Bianca Martens

Wageningen, 5 July 2019

Starch digestion kinetics in pigs

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and digesta passage behaviour

Bianca M.J. Martens

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Starch digestion kinetics in pigs

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Bianca M.J. Martens

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Abstract

The nutritional and energetic value of a pig's diet depend on the rate of starch digestion. At the moment, however, the reasons behind variation in *in vivo* digestion rates of different starches are not fully understood. The main aim of this thesis was to quantify the contribution of intrinsic starch structure, feed processing, and digesta passage behaviour on the kinetics and mechanisms of starch digestion in the upper gastrointestinal tract (GIT) of pigs.

The relation between intrinsic starch properties and *in vitro* digestion kinetics was studied in a large set of starches from various botanic origins. Across botanic sources, increased concentrations of A-type crystalline structure and short amylopectin side-chains increased hydrolysis rate. Within botanic sources, additional variation in *in vitro* hydrolysis kinetics was explained by other properties, such as the amylose content and the number of pores. Based on this *in vitro* work, three starch sources were selected (barley, maize, high amylose maize) and included each in three forms (isolated starch, ground cereals, extruded cereals) in experimental pig diets. Starch hydrolysis and disappearance was measured in the stomach and several parts of the small intestine, in addition to the rheological and physical behaviour and mean retention time (MRT) of digesta. Combining those results, an *in vivo* starch hydrolysis rate was determined for each diet. Consistent with our *in vitro* findings, the hydrolysis rate of starch in pigs was increased by extrusion and a decreased amylose content of maize starches. Starch originating from ground barley was fully hydrolysed in pigs, whereas 16% of starch ingested as ground maize was resistant to digestion.

Starch hydrolysis in the proximal small intestine was underestimated by our *in vitro* method (by 20% on average), whereas the amount of starch resistant to hydrolysis exceeded our *in vitro* predictions (by 9% on average). Consequently, glucose release from slowly digestible starch was less gradual than expected. Gastric bacteria were found to degrade granular starch in the stomach of pigs. Bacterial enzymes, extracted from stomach digesta, hydrolysed up to 29% of starch in a dynamic *in vitro* stomach model with a step-wise pH gradient from 6.5 to 2.0. Porcine salivary α-amylase, which has an optimum pH around 7.8, degraded 10% of gelatinized starch under these *in vitro* stomach conditions, but barely degraded any native starch.

The rate at which glucose, originating from starch, appears in the portal circulation does not only depend on the starch hydrolysis rate, but also on the transit time through the upper GIT. The MRT of digesta solids in the stomach of continuously fed pigs was longer (129 to 225 min) than in the small intestine (86 to 124 min). In addition, liquids remained around 60 min shorter in the stomach than digesta solids. Consequently, retention in the stomach will largely affect the appearance rate of glucose in the blood. The MRT in the stomach depended, in turn, mostly on the amount of water in stomach digesta as fraction of the theoretical maximum held by the digesta matrix.

In conclusion, the difference between the *in vitro* and *in vivo* situation is dominated by the initial rate of starch digestion, which was higher *in vivo* than *in vitro*. Gastric starch digestion and pre-digestion seem to contribute to the more rapid initial starch digestion *in vivo* and is a key factor in an accurate prediction of starch digestion rates.

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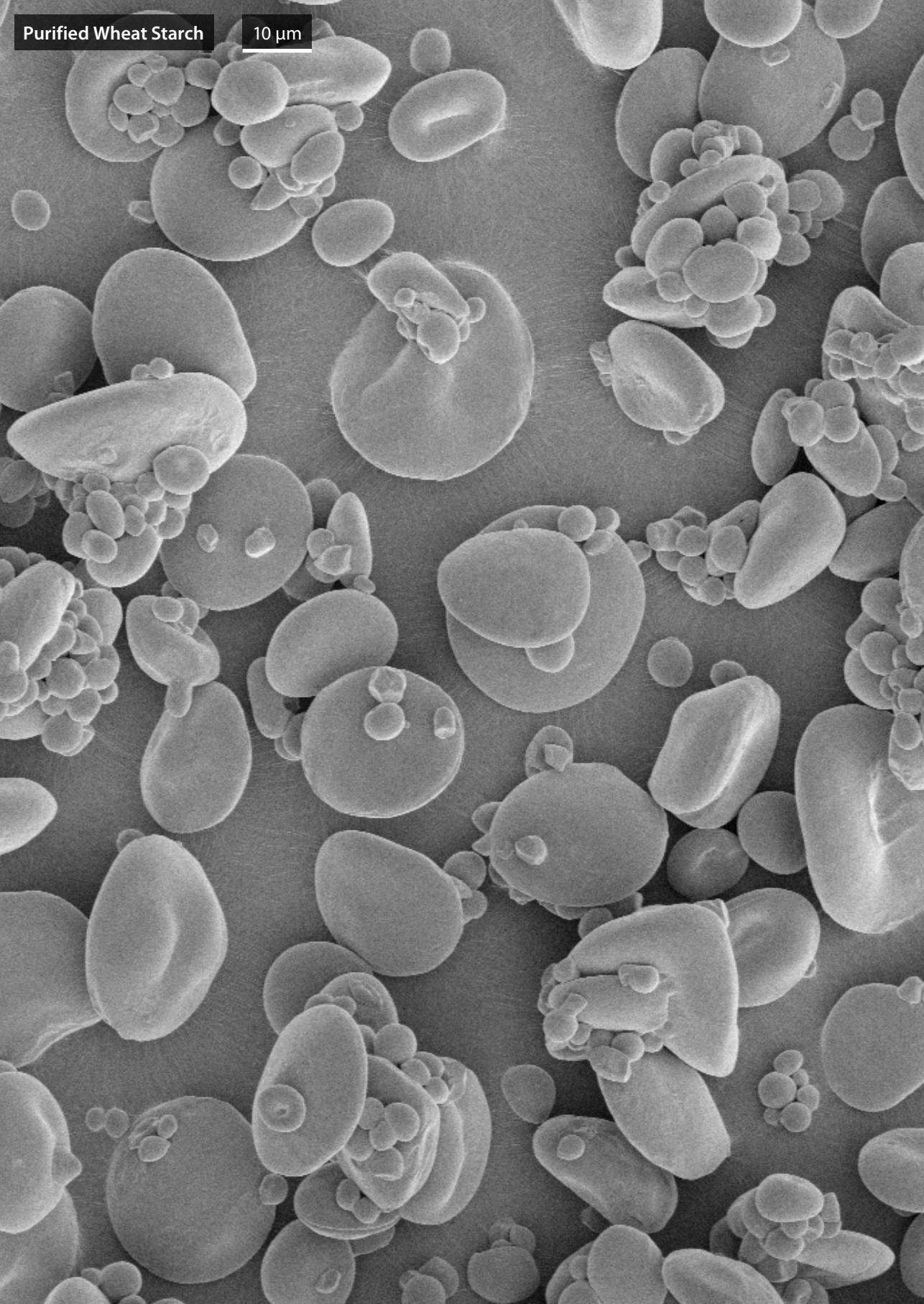
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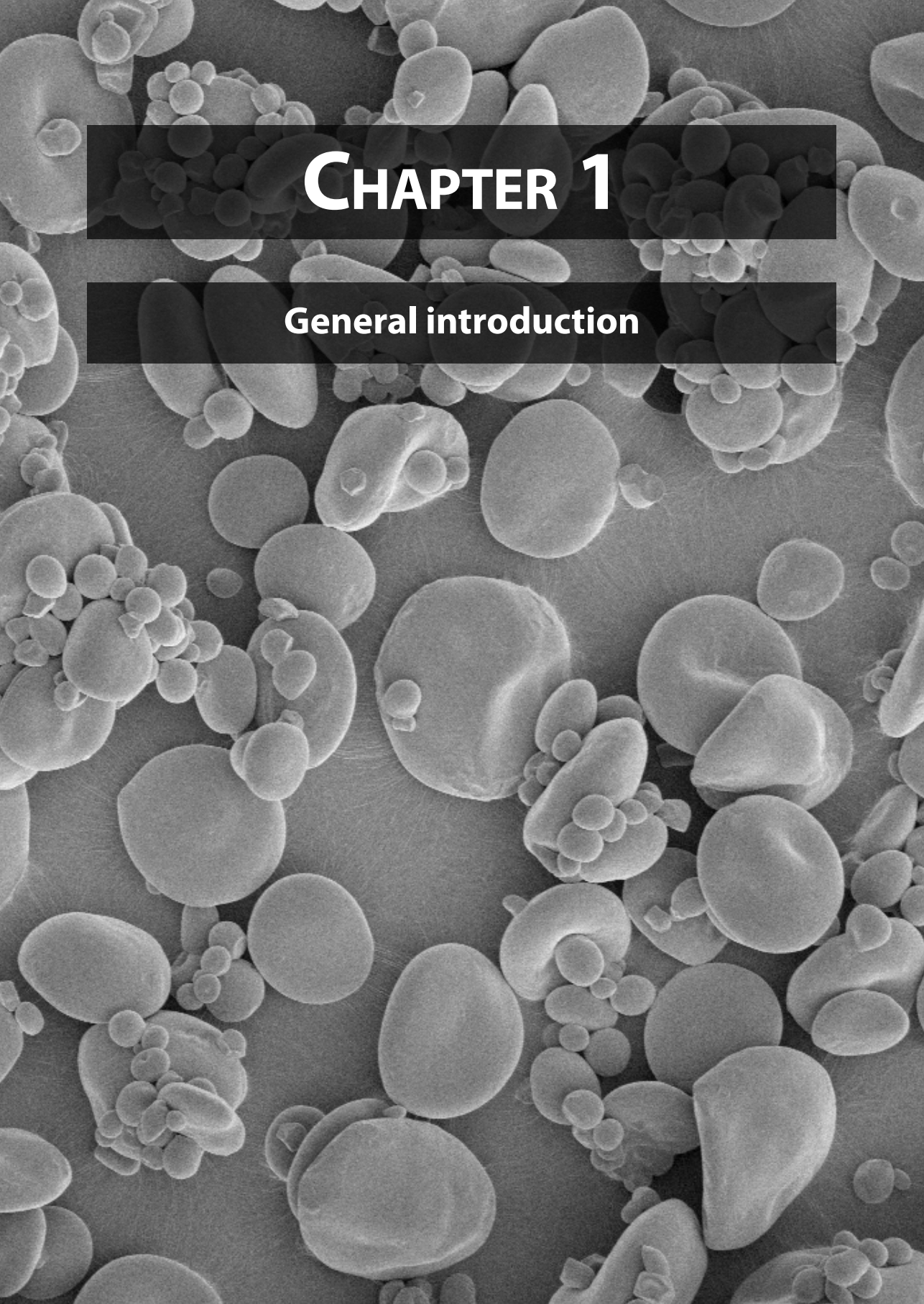

Chapter 1

General introduction

Background

The human population is expected to rise globally to 9.8 billion people by 2050[1]. This rise in population, combined with a rise in welfare, has an immense impact on global food security and is expected to lead to a 76% increase of annual meat production, equal to 197 million tonnes of meat, between 2012 and 2050[2]. Recent estimates are that livestock consumes globally 6 billion tonnes of feed per year at this moment, which includes one third of the global cereal production[3]. Currently, 86% of all animal feed consumed worldwide, is made of materials that are not edible by humans[3]. Feed consumed by monogastrics, however, consists for only 47% of materials that are not suitable for human consumption[3]. With a rapidly growing world population and a decrease in arable land per capita[2], one can easily imagine that the competition between arable land use for food and feed is expected to increase further in the near future.

To overcome the challenge of a global food or feed shortage, efficient use of recourses is crucial. Looking closely at animal feed, one can identify protein, fat, starch and fibre as the primary nutrients, which can either be used as fuel or as building blocks for animal source proteins. In the case of pigs, fuel is mainly provided as starch, which contributes to 60-70% of the total energy intake[4]. Therefore, being the dominant macronutrient and energy source, it is important to understand the process of starch digestion as well as its influence on the digestion of other nutrients.

The relevance of starch and its rate of digestion for pigs

The total tract digestibility of starch has long been considered as its only measure of nutritional value in feed evaluation systems. Gradually, this has shifted to the apparent ileal digestibility of starch, which is the amount of starch that is digested by endogenous enzymes of the pig before it reaches the end of the small intestine. The part of starch that is not digested at this point is referred to as resistant starch (RS). Starch digested in the small intestine is broken down to glucose, whereas RS is fermented by microorganisms, creating short chain fatty acids. Oxidation of short chain fatty acids is generally considered to yield less net energy for the pig than glucose from digested starch, which is why starch digestion in the small intestine is generally preferred over fermentation in the large intestine[5]. Microorganisms are also present in the small intestine, but in much lower numbers than in the large intestine[6]. Consequently, the contribution of small intestinal fermentation on starch degradation is generally neglected. In addition to differences in the extent of starch digestion, variation in botanic source and process conditions can result in differences in the rate of starch digestion. The *in vivo* rate of starch digestion depends on many factors, for example factors related to the animal, the dietary composition, and the passage behaviour of digesta in the animal. Those factors hamper the measurement of the starch digestion rate, as property of the starch itself, in the *in vivo* situation. Consequently, the rate of starch digestion is generally characterised based on *in vitro* methods.

In vitro classification of starch based on its digestion rate

One of the most commonly used *in vitro* methods is the one developed by Englyst and colleagues, which mimics the human stomach and small intestine[7]. In this method, starch hydrolysis is measured over time upon incubation with pancreatic α-amylase and fungal amyloglucosidase. Due to the large similarities between intestinal functions and absorptive processes of pigs and humans[8-10], the Englyst assay can also be used to predict starch digestion in pigs. Englyst et al. proposed three classifications of starch: Rapidly digestible starch (RDS) is the amount of starch digested within 20 minutes of *in vitro* incubation, slowly digestible starch (SDS) is the starch that is digested between 20 and 120 minutes, and resistant starch (RS) is the fraction that is not digested within 120 minutes[7]. At this moment, these categories are widely accepted and used to characterize starch digestibility.

Starch digestion rates and the utilization of other nutrients

Current feed evaluation systems estimate the nutritional value of a diet based on individual characteristics of each ingredients. The total nutritional value of a diet, however, may depend additionally on interactions between individual nutrients. For example, the *in vitro* rate of starch digestion can affect the utilization of protein. Protein consist of amino acids (AA), which are considered the building blocks of muscle tissue and can either be essential, meaning they should be supplied via the diet as animals can't synthesis them, or nonessential. Previous *in vivo* trials have shown that the net portal flux of essential AA is higher for pigs fed a diet rich in SDS, compared to a diet rich in RDS[11]. Asynchrony between the rates of glucose and AA appearance in the blood can also negatively affect protein utilization in restrictively fed pigs[12] and poultry[13]. The relation between the nutritional values of starch and protein partly depends on the energy requirements of the small intestine, which can be obtained from both glucose as well as AA[14,15]. Theoretically, a supply of glucose throughout the small intestine, upon ingestion of SDS, reduces the usage of AA as energy source in the distal small intestine.

In addition to the effect of starch digestion rates on protein, variation in the presence of starch in the large intestine can influence the degradation of fibres. For example, previous research showed that the presence of resistant starch alters the microbial composition and activity[16] and the enzyme profile[17] throughout the large intestine of pigs. This suggests that the presence of RS delays the fermentation of other fibres. Indeed, an increase in the RS content of a diet led to a reduction of the total tract digestibility of recalcitrant fibres in growing pigs[18].

Finally, the rate and extent of starch digestion affects the behaviour and energy utilization of animals. For example, feeding growing pigs a diet rich in RS resulted in lower activity-related energy losses[19]. Another study showed that RS rich diets increase meal durations inter-meal intervals[20], which can in turn lead to a lower daily energy intake when consuming lower levels of RS and SDS, compared to RDS[19]. Additionally, starch digestion rates affect animal performance. For example, broilers fed diets rich in SDS had a similar feed intake but greater

weight gain, resulting in a decreased feed conversion ratio (FCR), compared to birds fed RDS rich diets[13]. Another study showed that SDS rich diets can decrease the FCR of broilers while increasing feed intake[21]. The effect of starch digestion rate on animal performance, however, is not consistent across protein sources. For example, feeding broilers a rapidly digestible starch in combination with a rapidly digestible protein source, resulted in a lower average daily gain and average daily feed intake, compared with a rapid starch combined with a slow protein. Consequently, the FCR was lower for broilers fed a rapid starch combined with a slow protein, than when fed a rapid starch combined with a rapid protein. This effect on the FCR, however, was absent when feeding a slow starch, which resulted in equal average daily gains, feed intakes, and FCR when either combined with a slow starch or a rapid protein[22]. Overall, the energy efficiency and nutritional value of starch in the pig's diet, does not simply depend only on the extent of starch digestion, but seems an complex interplay between the rate of starch digestion and the presence of other nutrients.

PHYSICOCHEMICAL PROPERTIES OF STARCH

To understand variation in starch digestion kinetics, knowledge on the structure of starch is crucial. Despite a high homogeneity in its building block, solemnly glucose, starch is a complex molecule and has widely varying properties on different length scales. Several physicochemical properties of starch, which contribute to the final granule structure, will be discussed from small to large scale (left to right in **Figure 1.1**).

Molecular structure of starch

Starch is composed of two polysaccharides: Amylose, a linear α (1-4) chain of glucose molecules, and the much larger amylopectin, an α (1-4) linked glucan, which contains around 5% α (1-6) linkages, resulting in a branched molecule[23]. Starch normally contains about 20-30% amylose and 70-80% amylopectin, but amylose content can range from <1% in waxy starches to >80% in certain high amylose starches[24]. Amylose has a molecular weight of approximately 10^5 to 10^6 Daltons (Da), resulting in a degree of polymerisation (DP) of approximately 300-6000 glucose units[25-27]. Although amylose consists mainly of α (1-4) linked glucans, up to 0.5% of all

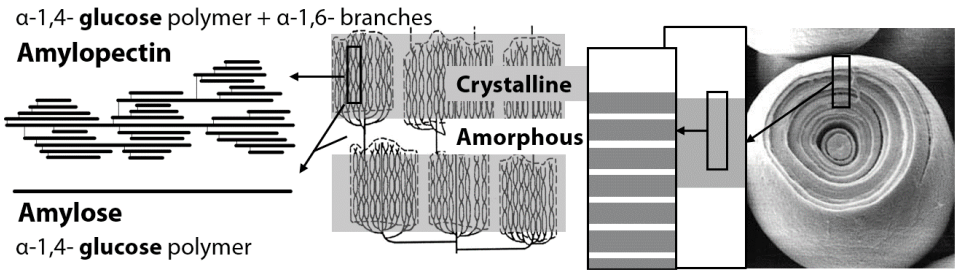

Figure 1.1. The complex structure of starch from molecular to granular organisation.

glucose units can be linked via an α (1-6) link, causing a small number of glucose branches on each amylose molecule[28,29].

Amylopectin has a molecular weight of 10^6 to 10^9 Da and a DP ranging from 9600 to up to 20 000[23,26]. Its side chains can be divided in A, B, and C chains (**Figure 1.2**), which are structured in dense crystalline clusters. A chains, with a DP between 6 and 12, are the smallest chains, which are found on the outside of the branched molecule and linked to a B chain[30]. B chains can be subdivided based on their length and number of crystalline clusters they participate in. The smallest B chains, B_1 chains, have a DP between 13 and 24. They are generally located on the outside of the amylopectin molecule and linked only to A chains. Consequently, those chains participate in only one cluster, whereas larger B chains participate in more clusters[31]. B chains are also linked to C chains, which are the largest chains, with a DP of at least 36 glucose units, and constitute the backbones of the amylopectin molecules[31,32].

Granular organisation and crystalline structure of the starch molecule

Clustered amylopectin side chains and amylose chains are organized in the helix conformation, which subsequently form crystalline lamella (9-10 nm)[33]. The crystalline structure of these lamella can be divided into three types, A-type, B-type, and C-type, based on their spatial organisation of the double helix. In A-type crystalline starch, glucose helixes are packed densely, whereas B-type crystalline starch is packed less dense in a hexagonal arrangement, leaving room for water molecules in between the helixes. Typically, cereal starches exhibit A-type crystalline structure and tubers B-type[24]. C-type crystalline starch consists of a combination of A- and B-type crystallinity[23,34]. This type of crystallinity is mainly observed in legume starches, high amylose cereal starches and tuber starches[34,35].

During starch biosynthesis, starch is deposited in crystalline shells or growth rings of 100 – 400 nm thick, which are made of alternated amorphous and crystalline lamella[36]. Ultimately, the lamellae form a water-insoluble granule[24]. The shape, size and distribution of granules varies highly between botanic sources[37]. For example, cereal starches are rather small and granules generally vary from 5 to 20 µm in diameter, whereas tuber and root starches can easily reach a granular diameter of 100 µm[38]. Granules also vary in their level of porosity and can have openings (pores) on the surface of the granule[39].

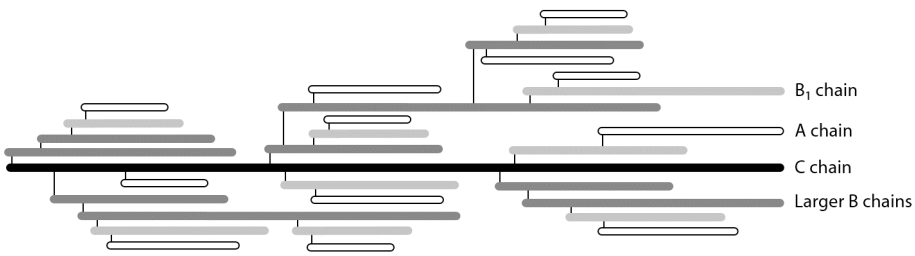

Figure 1.2. Simplified model of the molecular structure of amylopectin.

Other non-starch components associated with the starch granule

The starch granule is mainly composed of glucose polymers, however, up to 1.5% of the starch granule is made up of minor components such as lipids, protein and phosphorus[24]. Lipids associated with the starch granule are either located inside the starch granule or at the outside membrane of the granule. Lipids inside the granule are referred to as true starch lipids and consist mainly of lysophospholipids, whereas lipids present at the granule membrane are called surface lipids, which consist mostly of free fatty acids[40]. Also proteins identified in starch can be divided in those two categories: true proteins are present inside the granule, whereas surface proteins are present at the granule membrane[41].

STARCH GRANULES IN THE PLANT MATRIX

Starch is formed from CO_2 and H_2O during photosynthesis in plants and destined as energy source for various tissues throughout the plant. Typically, it is produced in excess and accumulated in storage organs of the plant, such as seeds, tubers, and roots, where it serves as energy supply during germination[42]. The main source of starch in conventional pig diets are cereals like barley, wheat and maize. Starch granules are present in the protein rich endosperm of the cereal seeds (**Figure 1.3**). The endosperm composition and structure depends on the amount and type of proteins, which typically classifies the endosperm in two types: a soft fraction with loosely packed starch, and a hard fraction in which starch is densely packed in the protein matrix[43,44]. The amounts of those fractions differ between botanic sources, but also within species of the same botanic source and even within a single cereal grain. The starch rich endosperm is surrounded by a cell wall that is composed of plant polysaccharides such as β-glucans and arabinoxylans. Similar to the protein composition of the endosperm cells, the composition of the cell wall varies greatly between botanic classes and cereal species[45].

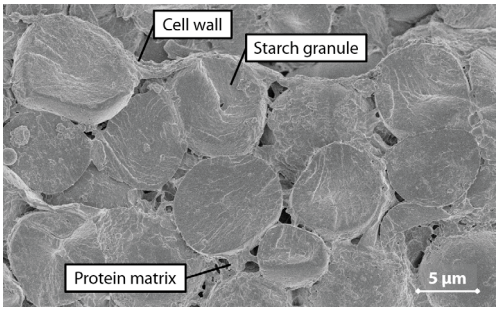

Figure 1.3. Scanning electron microscopic picture of the endosperm structure of maize (5000x magnified).

STARCH HYDROLYSIS MECHANISMS OF MONOGASTRICS ENDOGENOUS ENZYMES

In order for animals and humans to utilize glucose as energy source, starch needs to be hydrolysed to glucose monomers, which can be absorbed in their digestive tract. The hydrolysis of starch is performed by a combination of enzymes present in the gastrointestinal tract (GIT) of monogastrics.

Salivary α-amylase

Salivary α-amylase is excreted by salivary glands in the mouth of mammals and is the first starch-acting enzyme that is encountered upon ingestion. Due to the limited retention time of feed or food in the mouth, however, it is generally assumed that the contribution of salivary α-amylase on total starch digestion is limited in humans[46]. Not much is known about the contribution of porcine salivary α-amylase to starch digestion as characteristics of porcine salivary α-amylase are never intensively studied and reported. To speculate on the possible contribution of porcine salivary α-amylase to starch digestion, we can look into human salivary α-amylase, as human and pigs have a highly similar GIT with comparable intestinal functions and absorptive processes[8-10]. Human salivary α-amylase activity is optimal at pH 6.9, allowing for starch breakdown in the neutral environment of the mammalian mouth[47]. Additionally, salivary α-amylase might act upon starch during its residence in the stomach, although the stability and activity of salivary α-amylase in the stomach is still under debate[46-49]. Based on human salivary α-amylase, we can assume that the enzyme loses activity at pH values of 3.8 or lower in aqueous solutions[47,48], but that the presence of partly hydrolysed starch could offer protection to salivary amylase activity up pH values of around 2.5[49]. Accordingly, remainders of salivary α-amylase activity have been previously observed in the human small intestine[48].

Pancreatic α-amylase

Upon digesta transport from the stomach to the small intestine, digesta is mixed with pancreatic juice and starch comes into contact with pancreatic α-amylase. Pancreatic α-amylase is considered the main enzyme involved in starch digestion in monogastrics[46]. Structure wise, human pancreatic α-amylase is very similar to human salivary α-amylase as the amino-acid sequence of both enzymes is similar for 97%[51]. The function of porcine pancreatic

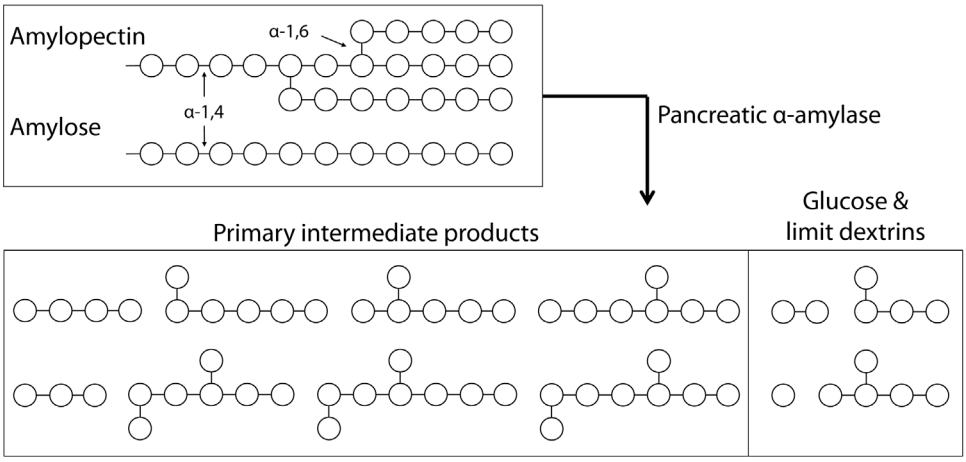

Figure 1.4. Primary intermediate products and end products (glucose and limit dextrins) formed by porcine pancreatic α-amylase upon incubation with amylopectin and amylose, adapted from[50].

α-amylase has been studied much more intensively than porcine salivary α-amylase and human pancreatic α-amylase. Upon starch digestion, porcine pancreatic α-amylase first attaches to the granular starch surface. Following, it binds part of the glucose molecule in its active side, which can host up to five glucose molecules. One of the α (1-4) linkages between the glucose molecules is then cleaved, which is most frequently the bond after the second, third, or forth glucose molecule[52]. Hydrolysis of amylopectin and amylose results in a series of maltodextrins, of which some (intermediate products) can be degraded further by α-amylase, whereas others (limit dextrins) are the end products of α-amylase hydrolyses (**Figure 1.4**). All linear α (1-4) maltodextrins with DP>4 are readily cleaved by the enzyme, whereas maltotetraose is somewhat resistant and maltotriose is too small to act as substrate. Hydrolysis of maltodextrins with DP5 results only in maltotriose and maltose, whereas all other linear α (1-4) maltodextrins result in multiple products, with maltose as the main reaction product[52].

Brush border enzymes

Breakdown products of pancreatic α-amylase are hydrolysed to glucose by a set of enzymes that are attached to the mammalian small intestinal membrane; the so-called brush border enzymes. Two enzyme complexes can be found anchored to the brush border epithelial cells of the small intestinal, which contribute to starch digestion: the sucrase-isomaltase complex (SIM) and the maltase-glucoamylase complex (MGAM). Both complexes consist of two subunits which are located on either the N-terminal site of the complex, which is closest to the small intestinal lumen, or the C-terminal site of the complex[53,54]. All subunits can readily cleave the α (1-4) link in the starch molecules. The α (1-6) linkages present in starches are mainly cleaved by N-terminal SIM, although α (1-6) hydrolysis is, in much lower rates, also reported for MGAM[55-57]. The substrate specificity of the four brush border enzymes differ greatly[58,59]. For example, N-terminal MGAM mainly releases glucose from short, linear gluco-oligomers only, whereas the other subunits can, in addition, digest longer and branched oligomers[60].
Despite decades of research, the exact role of brush border enzymes in starch digestion is still under debate. For example, the direct digestion of starch was reported for both maltase-glucoamylase and sucrase-isomaltase[61]. In addition, brush border enzymes might actually have activities beyond the membranous phase of the small intestine. For example, epithelial cells located on the tips of microvilli are budded off as brush border membrane vesicle[62], consequently, brush border enzymes can transit and diffuse to all parts of the intestinal lumen[63]. The exact extent of their contribution to the total rate of starch hydrolysis in mammals, however, remains unknown.

Rate and extent of starch digestion by the pig

Extent of starch digestion in pigs fed native starch

The extent of starch digestion in pigs is quantified as the amount of starch that is digested at end of the small intestine: the ileum. Two methods are commonly employed to quantify

Table 1.1. Ileal and feacal starch digestion coefficients (DC) for pigs fed raw starch.

Starch source[1]	Form	Mill used[2]	Sieve size	Starch (%)	BW start	Method[3]	Ileal DC	Fecal DC	Reference
Cereal starches									
Barley	Meal	-	-	41.0	78 kg	c	0.84	0.97	[71]
Barley	Meal	HM	4 mm	42.9	38 kg	c	0.93	1.00	[72]
Barley	Fine meal (0.7%>2mm)	-	-	-	-	-	0.97	-	[70]
Barley	Course meal (23.3%>2mm)	-	-	-	-	-	0.93	-	[70]
Barley HL	Meal	-	1 mm	41.0	40 kg	c	0.88	-	[73]
Barley HL	Meal	HM	1.6 mm	61.9	31 kg	c	0.85	1.00	[74]
Barley HL	Meal	HM	2.5 mm	27.3	6.1 kg	s	0.97	-	[75]
Corn	Meal	-	-	46.0	40 kg	c	0.97	-	[73]
Corn	Meal	HM	1.6 mm	61.9	31 kg	c	0.95	1.00	[74]
Corn	Meal	HM	1.6 mm	61.9	31 kg	c	0.99	1.00	[74]
Corn	Meal	HM	2.5 mm	32.0	6.1 kg	s	0.98	-	[75]
Corn	Meal	-	-	56.1	88 kg	s	0.89	1.00	[76]
Corn (0% AM)	Isolated starch			51.5	24 kg	s	0.96	1.00	[16]
Corn (80% AM)	Isolated starch			67.0	7.1 kg	s	0.78	1.00	[68]
Corn (85% AM)	Isolated starch			51.5	24 kg	s	0.88	0.94	[16]
Wheat	Isolated starch			58.4	45 kg	c	0.96	1.00	[77]
Wheat	Isolated starch			58.4	45 kg	c	0.96	1.00	[77]
Wheat	Meal	DM + RM		65.2	40 kg	c	0.99	1.00	[78]
Wheat	Meal	-	1 mm	46.0	40 kg	c	0.95	-	[73]
Wheat	Meal	HM	1.6 mm	59.5	31 kg	c	0.98	1.00	[74]
Oats	Meal	DM		57.8	40 kg	c	0.97	1.00	[79]
Oats	Fine meal (<350 mu)	DM		59.8	40 kg	c	0.99	1.00	[79]
Oats	Meal (350-630 mu)	DM		55.5	40 kg	c	0.99	1.00	[79]
Oats	Course meal (>630 mu)	DM		50.8	40 kg	c	0.99	1.00	[79]
Oats	Dry rolled	RM		60.0	40 kg	c	0.97	1.00	[78]
Oats HL	Meal	HM	1.6 mm	63.0	31 kg	c	0.97	1.00	[74]

Table 1.1. (Continued) Ileal and feacal starch digestion coefficients (DC) for pigs fed raw starch.

Starch source[1]	Form	Mill used[2]	Sieve size	Starch (%)	BW start	Method[3]	Ileal DC	Fecal DC	Reference
Cereal starches									
Sorghum	Meal	HM	3.2 mm	-	30 kg	c	0.86	0.99	[69]
Sorghum	Meal	HM	6.4 mm	-	30 kg	c	0.78	0.95	[69]
Sorghum	Dry rolled	RM		-	30 kg	c	0.72	0.96	[69]
Sorghum	Meal	HM	1.6 mm	60.5	31 kg	c	0.98	1.00	[74]
Rye	Meal	HM	1.6 mm	55.7	31 kg	c	0.92	1.00	[74]
Rice (HL)	Meal	HM	1.6 mm	73.8	31 kg	c	0.99	1.00	[74]
Legume starches									
Pea	Meal	-	-	31.2	34 kg	c	0.86	-	[80]
Pea	Meal	-	-	35.0	34 kg	c	0.85	-	[80]
Pea	Meal	-	-	30.8	34 kg	c	0.87	-	[80]
Pea	Meal	-	-	35.6	34 kg	c	0.87	-	[80]
Pea	Meal	HM	4 mm	38.1	38 kg	c	0.79[4]	0.99[4]	[72]
Faba beans	Meal	-	-	22.7	34 kg	c	0.82	-	[80]
Faba beans	Meal	-	-	22.2	34 kg	c	0.86	-	[80]
Faba beans	Meal	HM	3 mm	43.6	35 kg	c	0.95	1.00	[81]
Faba beans (HL)	Meal	HM	3 mm	45.5	35 kg	c	0.98	1.00	[81]
Tuber starches[5]									
Potato	Isolated starch			47.5	38 kg	c	0.40[4]	0.98[4]	[72]

[1] Origin of starch, specified whether starch originated from hull-less (HL) material or when the starch had an a-typical amylose (AM) content.
[2] The type of mill used to grind the raw material: Hammer mill (HM), disc mill (DM), or roller mill (RM).
[3] Method used for digesta collection: the slaughter (s) or cannulation (c) method.
[4] Average over two collection periods.
[5] Limited *in vivo* studies have been performed with tuber starch, as tubers contains very high levels of RS and could potentially disturb digestion processes.

digestible starch: the first is the slaughter technique, which involves slaughtering of an animal at a given time point after feeding and the collection of a digesta sample from the pigs' ileum. The second method is the cannulation technique, which consists of the surgically implantation of a permanent cannula in the ileum. Both methods depend on the usage of an indigestible tracer. These compounds are usually metal complexes such as titanium oxide, chromium EDTA, or chromium chloride, which are assumed to be not degraded or absorbed by the animal and have a flow behaviour in the GIT that is comparable with starch[64]. By measuring the recovery of starch and tracer in each GIT segment, one can calculate the digestibility of the starch.
An overview on the ileal and faecal digestibility of raw starches, either fed as isolated starch or ground product, is provided in **Table 1.1**, as measured with the slaughter (s) or cannulation (c) method in pigs. Generally, starches from cereal origin are almost completely digested at the ileum, whereas starches from legume or tuber origin are more resistant to digestion. The difference in starch digestibility between cereal, legume, and tuber starches is related to their crystalline structure, being A-type for cereal starches, C-type for legume starches, and B-type for tuber starches[65]. Due to the hydrogen bonds with water molecules present in B- and C-type starch crystals, these starches are more resistant to digestion than the A-type starch[66,67]. Within cereal starch, starches with a high level of amylose are more resistant to digestion[16,68]. In addition, starch digestion can be hampered by the cellular plant matrix. Therefore, grinding into a small particle size can increase starch digestion[69,70]. This effect is, however, not always observed when comparing ileal starch digestibility levels across studies, suggesting additional effects of other aspects, such as difference in the age and breed of pigs, or the variety of cereal used.

Effects of hydrothermal processing on ileal starch digestibility in pigs

In common pig diets, starch is not provided raw, but in a processed form. Typical processing techniques involve combinations of mechanical force, shear force, heat, and pressure, which are applied to the raw feedstuff. Pelleting and extrusion are examples of widely used processing techniques in the western pig feed industry. During the pelleting process, feedstuff are conditioned by steam injection and subsequently forced through a die, typically causing the product temperature to reach levels between 60 and 90°C[82-84]. During this process, the combination of heat and shear causes partly gelatinization of starch and a limited reduction of particle size[82,83]. During extrusion, the conditions applied to the feedstuff are more severe than during pelleting, leading generally to a greater change in physicochemical properties. Commonly used processing methods and their effect on starch digestion are summarized in **Table 1.2**. Starch gelatinization, caused by heat and shear pressure, causes a decrease in crystalline structure of starch, making it much easier digestible for pancreatic α-amylase. Typically, starch digestibility is increased most by intense processing conditions, such as extrusion and toasting. This increase is especially pronounced in legume and tuber starches[72,81].

Table 1.2. Ileal and faecal starch digestion coefficients (DC) of pigs fed processed starch.

Starch source[1]	Processing	Product T[2]	Starch (%)	BW start	Method[3]	Ileal DC	Faecal DC	Reference
Cereal starches								
Barley, 30% AM	Pelleted	80°C	53.6	37 kg	c	0.97		[84]
Barley, 30% AM HL	Pelleted	80°C	56.9	37 kg	c	0.98		[84]
Barley, 40% AM	Pelleted	80°C	47.7	37 kg	c	0.93		[84]
Barley, 9% AM	Pelleted	80°C	50.2	37 kg	c	0.96		[84]
Barley, 9% AM HL	Pelleted	80°C	53.6	37 kg	c	0.98		[84]
Barley	Extruded	155°C	38.1	78 kg	c	0.97	1.00	[71]
Barley	Extruded	145°C	42.4	38 kg	c	0.99	1.00	[72]
Barley	Steam-cooked	99 °C	27.3	6.1 kg	s	0.98		[75]
Maize	Steam-cooked	99 °C	32.0	6.1 kg	s	0.99		[75]
Maize, 85% AM	Hydrothermal treated	125°C	51.5	24 kg	s	0.70	0.83	[16]
Legume starches								
Pea	Extruded	145°C	38.3	38 kg	c	0.92[4]	1.00[4]	[72]
Pea	Dried	-	45.0	35 kg	c	0.93	1.00	[85]
Pea	Toasted	130°C	46.4	35 kg	c	0.94	1.00	[85]
Faba beans HL	Steamed + pelleted	103°C	46.0	35 kg	c	0.94	1.00	[81]
Faba beans	Extruded + pelleted	140°C	43.0	35 kg	c	0.98	1.00	[81]
Tuber starch								
Potato	Extruded	145°C	50.1	38 kg	c	0.98[4]	1.00[4]	[72]

[1] Origin of starch, specified whether starch originated from hull-less (HL) material or when the starch had an a-typical amylose (AM) content.
[2] Maximum product temperature reached during processing.
[3] Method used for digesta collection: the slaughter (s) or cannulation (c) method.
[4] Average over two collection periods.

Rate of starch digestion in pigs

In vivo digestion rates of starch can be studied via the concentration and flux of glucose in the portal vein. Consequently, placing a catheter in the portal vein has become a popular method to study *in vivo* starch digestion kinetics. With this technique, a lower initial, but longer lasting glucose appearance was observed after feeding pigs a diet with pea starch, compared with maize starch[11]. Portal vein glucose concentrations can also be used to study the feed matrix effect. For example, an increased diet viscosity, obtained via the inclusion of 3-6% β-glucan, decreased portal glucose flux during the first 60 minutes after pigs were fed diets with wheat starch[89].

Another technique to study starch digestion kinetics, is by analysing the undigested starch remaining in different subsections of the small intestinal after slaughter of a pig. Only a limited number of starch sources have been studied in this way, which are summarised in **Table 1.3**. In the pig, C-type crystalline starch seems to be digested slower than A-type starch and isolated starches seems to be digested faster than ground cereals. The coefficient of starch digestion, however, varies widely between studies and has physiological unrealistic values in some GIT compartments. For example, most studies report a negative starch DC in the stomach, which could only be caused by a different flow behaviour of the tracee compared with the tracer.

In addition to the limited number of *in vivo* studies, *in vitro* models have been used widely to study the effect of starch characteristics on variation in digestion kinetics. For example, starches rich in A-type crystalline structure are more rapidly digestible than starches rich in B-type[90], starches with an increased amylose content are digested slower than low-amylose

Table 1.3. Digestion coefficients (DC) of starch, measured in the stomach and various segments of the small intestine of pigs.

Starch		Digestion Coefficients						BW start	Source
Source	Form[1]	Stomach	Small intestine[2]				Faecal		
Rye	Meal		0.07	0.33	0.60	0.82	0.97	11 kg	[86]
Barley	Meal (HL)		-0.42	0.06	0.49	0.74	0.96	11 kg	[86]
Oat	Meal (<350 mu)	0.00	0.64	0.95	0.98	0.99		40-50 kg	[79]
Oat	Meal (>630 mu)	-0.10	0.49	0.90	0.98	0.99		40-50 kg	[79]
Maize	Gelatinized starch	0.15	0.63	0.86	0.99	0.98	1.00	40-45 kg	[87]
Pea	Isolated starch	-0.09	-0.04	0.80	0.98	0.98	1.00	40-45 kg	[87]
Wheat	Isolated starch (L)	-0.08	0.63	0.95	0.99		1.00[3]	60 kg	[88]
Wheat	Isolated starch (S)	0.21	0.51	0.87	0.98		0.99[3]	60 kg	[88]
Wheat	Gelatinized starch	0.24	0.50	0.86	0.99		1.00[3]	60 kg	[88]
Tapioca	Isolated starch	-0.02	0.64	0.76	0.84		0.99[3]	60 kg	[88]

[1] Form of starch, specified whether starch originated from hull-less (HL) material and isolated wheat starch consists of small (S) or large (L) granules.

[2] Starch DC are measured in four segments of equal length[86], three segments of equal length[88], or in three segments of equal length and in the ileum via ileal cannulation[79,87].

[3] Measured in caecum.

starches[91-94], and starches which have longer amylopectin side chains correlate with a slower digestibility[95,96]. In addition, smaller granules are generally digested faster than bigger ones, due to the larger surface area for enzymes to act upon[97,98]. The presence of pores on the starch granule allow enzymes to digest starch granules from the inside out, which also leads to a more rapid digestion[39,99]. The contribution of each distinct starch characteristic, however, is not fully understood yet, as variation in intrinsic starch characteristics is inherently correlated with variation in the botanic starch source[100].

Identification of knowledge gaps

New insights in the relation between starch digestion and pig performance caused a shift from research on the extent of starch digestion, to the rate of starch digestion. *In vitro*, the relation between starch properties and digestion rate is studied extensively, but not fully understood yet[101]. The prediction of starch digestion rate based on starch characteristics is hampered by inherent combinations of these starch characteristics within each botanic source. The quantitative contribution of each individual starch characteristic is lacking, especially across botanic sources that are relevant for pig nutrition.

In the meantime, *in vitro* predictions of starch digestion kinetics are widely used to study the effect of variation in starch digestion kinetics on pig performance. Soon after Englyst's characterization of starches into RDS, SDS, and RS[7], the same team identified a correlation between RDS and the glycaemic index in humans[102,103]. Consequently, starches rich in *in vitro* determined concentrations of RDS were presumed to be rapidly digestible in the small intestine of monogastrics. Englyst's characterization has ever since widely been used to study the effects of starch digestion rates on animal performance and health. Subsequently, research has focussed on the relation between *in vitro* digestion rates and starch structure. The *in vivo* digestion rate in relation to starch structure, however, is much less studied and remains rather unknown[104,105]. Evidence that *in vitro* starch digestion correctly predicts the *in vivo* situation for pigs is limited. Not only has an insufficient number of studies been performed on starch disappearance in the small intestine, evaluation of starch digestion kinetics is hampered by missing information on digesta passage rate. At the moment, glucose appearance in the portal vein is the most accepted method to study starch digestion rates. Glucose recovery in the portal vein, however, does not provide insight in the breakdown mechanisms of starch and the contribution of digesta passage behaviour inside the GIT. Additionally, the total glucose recovery in the portal vein is generally lower than the ileal disappearance of starch[14,106-108]. Consequently, the fate of starch in the digestive tract is partly unknown, of which the extent varies widely between studies.

Aim and outline of this thesis

The main aim of this thesis was to quantify the contribution of intrinsic starch structures, feed processing, and digesta passage behaviour on the kinetics and mechanisms of starch digestion

in the upper GIT of pigs (**Figure 1.5**). To this end, we first studied the relation between intrinsic starch properties and *in vitro* digestion kinetics in a large set of starches from various botanic origins, attempting to uncouple the effects of intrinsic starch properties from that of botanic origin. The results of this work are described in **Chapter 2**. Based on this *in vitro* work, we selected three starch sources, which were each included in three experimental diets using different forms of processing. The resulting nine dietary treatments were frequently fed to growing pigs to reach a steady state. After slaughter, starch break down and disappearance was measured in several parts of the small intestine, as described in **Chapter 3**. In the same animal trial, we measured digesta passage rate in the stomach and small intestine. The research presented in **Chapter 4** revealed how these passage rates relate to physical and rheological properties of diets and digesta. In addition to gastric emptying of pigs in a steady state, the study presented in **Chapter 5** targeted the effect of feed processing on gastric emptying of meal-fed pigs, measured with the non-invasive ^{13}C breath test. Additionally, digesta passage rate described in chapter 4, is used to estimate *in vivo* kinetics of starch digestion as presented in chapter 3. The kinetics of starch digestion in the small intestine made us reconsider the effect of the stomach on starch digestion. In **Chapter 6**, we describe our study on the fate of starch in a dynamic stomach model. Therefore, we used both enzymes extracted from bacteria present in the stomach, as well as porcine salivary amylase. Finally, the general discussion presented in **Chapter 7** provides an overview and future perspectives regarding starch digestion kinetics throughout the upper GIT of the pig, combining the results described in this thesis with existing literature.

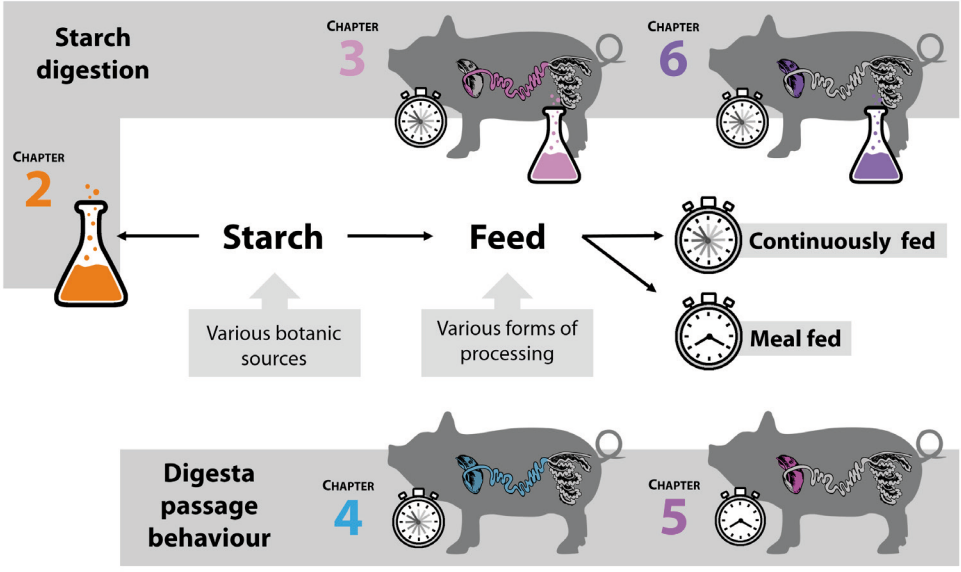

Figure 1.5. Visual outline of this thesis: Research presented in chapters 2, 3, and 6 focuses on starch digestion, whereas research in chapters 4 and 5 focuses on digesta passage behaviour. Results presented in chapter 2 originate solemnly from *in vitro* experiments, whereas other chapters include results from *in vivo* studies.

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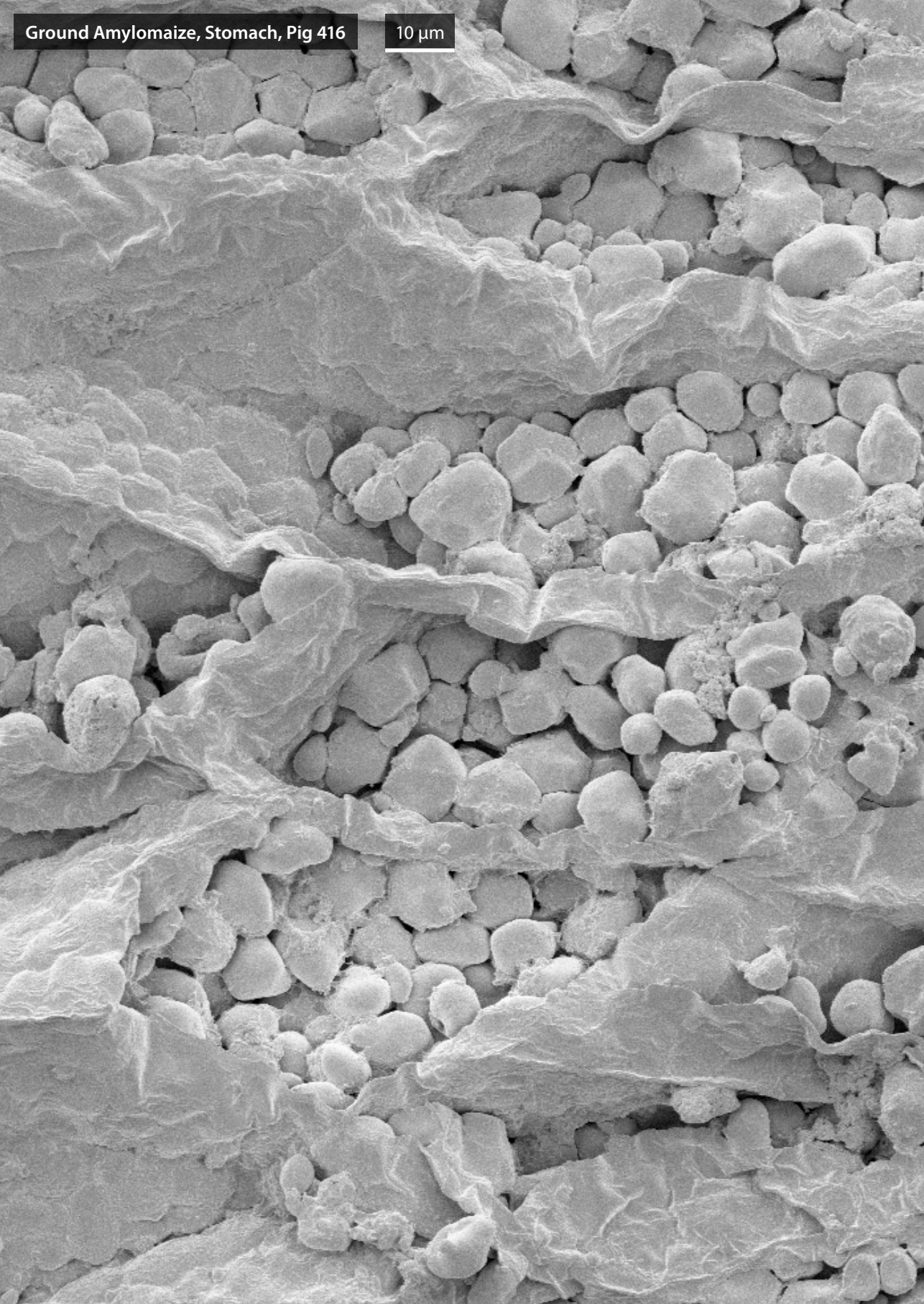
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Chapter 2

Amylopectin structure and crystallinity explains variation in digestion kinetics of starches across botanic sources in an *in vitro* pig model

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Abstract

Starch is the main source of energy in commonly used pig diets. Besides effects related to the extent of starch digestion, also several effects related to variation in digestion rate have recently been demonstrated in non-ruminants. Different rates of starch digestion in animals and in *in vitro* models have been reported, depending on the botanic origin of starch. Starches from different botanic sources differ widely in structural and molecular properties. Predicting the effect of starch properties on *in vitro* digestion kinetics based on existing literature is hampered by incomplete characterization of the starches, or by a selective choice of starches from a limited number of botanic sources. This research aimed to analyse the relationships between starch properties and *in vitro* digestion kinetics of pure starches isolated from a broad range of botanic origins, which are used in non-ruminant diets or have a potential to be used in the future. Therefore we studied starch digestion kinetics of potato, pea, corn, rice, barley, and wheat starches, and analysed the granule diameter, number of pores, type and amount of crystalline structure, amylose content and amylopectin side-chain length of all starches.
Multivariate analysis revealed strong correlations among starch properties, leading us to conclude that effects of most starch characteristics are strongly interrelated. Across all analysed botanic sources, crystalline type and amylopectin chain length showed the strongest correlation with *in vitro* digestion kinetics. Increased percentages of A–type crystalline structure and amylopectin chains of DP 6-24 both increased the rate of digestion. In addition, within, but not across, (clusters of) botanic sources, a decrease in amylose content and increase in number of pores positively correlated with digestion kinetics.

Introduction

Starch is the main source of energy in commonly used diets for non-ruminants. Starches in those diets are of various botanic origin, which usually causes variation in digestion rate in the gastrointestinal tract. Diverse effects related to variation in starch digestion kinetics have been demonstrated in pigs. For example, diets containing resistant starch (RS) or slowly digestible starch (SDS) affect feeding patterns[1] and energy partitioning[2] when compared to diets with rapidly digestible starch (RDS). Also an asynchrony between the rates of glucose- and amino acid absorption negatively affects protein utilization in pigs[3] and poultry[4].

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[5]. Starch normally contains about 20-30% amylose and 70-80% amylopectin, but amylose content can range from <1% in waxy starches and >70% in certain high amylose starches[6]. The branched amylopectin molecule contains regions with low and high levels of branches. In highly branched regions, side-chains of amylopectin are grouped, forming crystalline zones (clusters)[7]. Side chains of the amylopectin molecule can be divided in A, B, and C chains. C chains constitute the backbones of the amylopectin molecules, to which B-chains are linked that at the same time carry one or more branches. B chains are given additionally a number based on their participation in side chain clusters. B_1 chains participate in one cluster, B_2 and B_3 chains participate respectively in two or three clusters. A chains are present at the outside of the branched molecule and have only an α(1-6) linkage to B_1 chains[8]. Based on the cluster model of Robin et al.[9] and on the study of Hanashiro et al.[10], A chains are believed to correspond with side chains with a degree of polymerisation (DP) of 6-12, B_1 chains with DP 13-24, B_{2-4} chains with DP 25-36, and B_{5-x}-and C-chains with DP>36. Clustered amylopectin side chains and amylose chains are organized in the helix conformation that subsequently form crystalline structures, which can be divided into three types: A, B and C. In A-type crystalline starch, glucose helixes are packed densely while B-type crystalline starch is packed less dense, leaving room for water molecules in between the branches. C-type crystalline starch consists of a combination of A- and B-type crystallinity[5]. During starch biosynthesis, starch is deposited in alternating amorphous and crystalline shells (growth rings), 100 – 400 nm thick[7], ultimately resulting in a water-insoluble granule[6]. The shape, size and distribution of granules varies highly between botanic sources[11]. Granules also vary in the level of porosity and can have openings (pores) on the surface of the granule[12].

Research has been conducted to describe relationships between structural starch features and (*in vitro*) digestibility. However the outcome of research is influenced by the choice of starting material. For instance, when analysing purified starch samples, which originate from the same botanic source, high levels of amylose correlate positively with the proportion of *in vitro* measured RS[13-16]. This is supported by several *in vivo* studies, which have shown a negative correlation between amylose content and the blood glucose response[17,18] and consequently the glycaemic index[19]. Furthermore, amylopectin affects digestibility within botanic sources

as well, as longer amylopectin side chains correlate with a slower digestibility[20,21]. Across botanic sources, it is believed that a higher proportion of crystallinity correlates with more RS, whereas a higher amorphous fraction results in a lower rate of digestion[14,22,23] even though amorphous and crystalline regions are assumed to be digested simultaneously[24]. Due to the water molecules present in B- and C-type starch crystals, these starches are usually digested slower than the A-type starch[24,25]. The size of the starch granule is also believed to correlate negatively with the rate of starch digestion across botanic sources. Smaller granules are digested faster than bigger ones, which is generally believed to be caused by a larger surface area on which enzymes can act[26,27]. Lastly, the presence of pores is proven to affect starch digestion; due to these channels, enzymes are supposed to digest starch granules from the inside out, leading to a more rapid digestion[12,28].

Estimating the contribution of each distinct characteristic to starch digestion kinetics is hampered by inherent combinations of these starch characteristics within each botanic source. For example, large granules are usually digested slower than small granules, but often consist of slowly digestible B-type crystals[27]. The same goes for amylose content and crystalline structure, as high amylose cereal starches are mostly reported to consist of C- or B-type crystalline structure, in contrast to A-type crystalline structure, which is typical for low amylose cereal starch[29-31]. Furthermore, amylopectin molecules with longer chains are correlated with more B-type crystals[20]. Also correlations between amylose content and granule size[32] and amount of crystalline structure[14] have been reported before.

Quantifying the influence of each individual starch characteristic based on literature is extremely difficult[33]. By relating all characteristics to the *in vitro* digestion kinetics of those starches, we aimed to identify, across botanic sources, the starch characteristics that are affecting starch digestion most in starches relevant for non-ruminant feed production. In this study we measured the following starch characteristics: type and amount of crystalline structure, amylose content, amylopectin chain length, granule size and the number of pores of starches from a wide range of botanic sources. The selected botanic sources are commonly used in animal feeds, or have a potential for future use. The purpose of this study was to correlate intrinsic starch properties to starch digestion kinetics. Consequently, we selected purified starches to eliminate effects of other feed related components.

Materials and methods

Starches

Pure starches from different botanic origins were selected to cover maximum relevant variation in amylose content, granular size, proportion and type of crystallinity and the presence of pores. 15 different starches were used, of which one starch was additionally sieved into 5 fractions, creating a total of 20 samples. Rice starches Remyline AX-DR (*waxy rice*), Remy B7 (*rice A*) and Remy B (*rice B*) were kindly provided by the Beneo group (Tienen, Belgium). Nastar

yellow pea starch (*pea A*) was obtained from Cosucra group (Perq, Belgium) and wheat starch was obtained from Fluka Biochemika (Buchs, Switzerland). Corn starches M03401 (*corn A*) and M04201 (*waxy corn*) were kindly provided by Cargill B.V. (Vilvoorde, Belgium), high amylose corn starch Hylon V (*high amylose corn A*) was obtained from Ingredion (Westchester, IL, U.S.A.), and barley starch was kindly provided by Altia Corporation (Helsinki, Finland). Native potato starch, Eliane 100 (*waxy potato*), and experimental Heat Moisture Treated potato starch (*HMT potato*), based on native potato starch, were kindly provided by Avebe (Veendam, The Netherlands). Regular corn starch (*corn B*) and high amylose Amylomaize (*high amylose corn B*), and regular pea starch (*pea B*) were obtained from Roquette (Nord-Pas-de-Calais, France). All starch samples were stored at room temperature until analysed. All enzymes and chemicals were purchased from Sigma Aldrich (Saint Louis, MO, U.S.A.) unless stated otherwise.

Physical and chemical analyses

Granule size distribution was determined as the mean of 5 measurements (maximum standard deviation of the mean diameter was 0.41 µm) with a Mastersizer3000 (Malvern Instruments, Malvern, U.K.). Potato starch samples were fractionated using a vibratory sieve shaker (AS200 digit; Retsch GmbH & Co., Haan, Germany) with demineralized water as washing liquid. Size fractions were air-dried overnight at 40°C. Potato starch was separated into five fractions: smaller than 20 µm, 21-32 µm, 33-53 µm, 54-71 µm, and 72-109 µm, of which the granule size distribution was determined as described above.

Starch morphology was determined with a Scanning Electron Microscope (SEM) (Magellan 400, FEI, Eindhoven, the Netherlands). Dry starch granules were attached on sample holders using carbon adhesive tabs (EMS, Washington, United States) and sputter coated with 15 nm tungsten (EM SCD 500, Leica, Vienna, Austria). Granules were analysed with a field emission SEM with SE detection at 2 kV. Starch morphology was studied at 1000 times magnification and starch surfaces were studied at 25000 times magnification. All samples were measured at the Wageningen Electron Microscopy Centre and from each sample at least 20 individual granule surfaces were studied. To ensure the analysis of a homogenous sample, individual granules were selected in such a way that granules from each diameter were represented in accordance with their relative abundance as measured with the Mastersizer.

X-ray diffraction (XRD) was used to identify the crystalline structure of starch samples. Wide angle X-ray scattering (WAXS) powder diffractograms were recorded on a Bruker Discover D2 diffractometer (Bruker corporation, United States) in the reflection geometry in the angular range 4-33°(2θ), with a step size of 0.02°(2θ) and an acquisition time of 2.0 s per step. The diffractometer was equipped with an LINEXEYE™ Silicon-strip detector, which had a 4° - 5° active area. The Co Kα1 radiation (λ = 1.7902 Å; X-ray tube is air cooled) from the anode, generated at 30 kV and 10 mA, was monochromatized using a Ni filter. The diffractometer was equipped with a 1 mm divergence slit and a 0.5 mm knife edge above the sample stage, which enabled accurate measurements from 4°(2θ) upwards. The proportion of crystallinity was determined by subtracting the background from the WAXS pattern of a sample. Intensities

are expressed as relative where the intensity of the peak at diffraction angle 20.0°(2θ) is set to 1.0, as this peak showed the highest intensity in both A- as B-type of starch. Samples that contained a mixture of typical A- and B-type crystalline diffraction spectra were modelled (with a least-squares error fit procedure) to deduce the exact proportions of A- and B-type crystallinity.

Amylose content of starch samples was determined according to the amylose/amylopectin assay procedure of the supplier (Megazyme, Wicklow, Ireland, in which amylose was separated from amylopectin by Concanavalin A lectin. Amylose was enzymatically hydrolysed and glucose recovery was determined by a glucose oxidase peroxidase reaction. All samples were analysed in triplicate (maximum standard deviation of 0.8%).

Total starch content of each sample was determined according to the total starch assay procedure from Megazyme by subsequently washing the starch with ethanol (part e of the assay procedure supplied by Megazyme), dissolving the starch in KOH (part c of the assay procedure supplied by Megazyme) and finally enzymatically hydrolysing the starch (part a of the assay procedure supplied by Megazyme). Glucose was determined with a glucose oxidase peroxidase reaction. The total starch content was solemnly used as a correction factor in the *in vitro* digestion assay. All samples were analysed in triplicate (maximum standard deviation of 1.1%).

Amylopectin chain length was determined after starch samples were debranched with 0.32 mL isoamylase (500 units/mL, Megazyme) per gram starch at pH 4 for 17 hours at 50 °C, after starch samples were dissolved in boiling water. Debranched samples were analysed on ICS5000 High Performance Anion Exchange Chromatography system with Pulsed Amperometric detection (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 mol/L NaOH and (B) 1 mol/L NaOAc in 0.1 mol/L NaOH and the column temperature was 20 °C. The elution profile was as follows: 0–50 min 5–40% B, 50–65 min 40–100% B, 65–70 min 100% B, 70–70.1 min 100–5% B and finally column re-equilibration by 5% B from 70.1 to 85 min. The injection volume was 10 µl. Glucose was used to quantify concentrations of side-chains with DP up to 5 (although this fraction made up <0.1% of the amylopectin molecule) and maltohexaose was used to quantify side-chains with DP>5. All samples were analysed in duplicate and the average amylopectin chain length per sample had a maximum standard deviation of 0.7 DP. To facilitate the comparison of starch samples, amylopectin side-chains were clustered into four categories, DP 6-12, DP 13-24, DP 25-36, and DP>36, in analogy to the cluster model of Robin et al.[9]. For Pearson's correlation procedure and PCA, variations in side-chain length of amylopectin were summarized in the ratio between short (DP 6-24) to long (DP>36) side-chains. "Short" chains reflect A and B amylopectin side-chains that are involved in only 1 cluster, whereas "long" chains reflect B and C chains involved in 5 or more clusters, respectively[9,10].

In vitro starch digestion

In vitro starch digestion kinetics were determined with a digestion method described by Englyst et al.[34] and Van Kempen et al.[35]. 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, followed by incubation in a sodium acetate buffer (0.5 mol/L) containing porcine pancreatin (P-7545), amyloglucosidase (A7095) and invertase (I4504) at pH 6 and 39°C for 360 min. In comparison to the assay described by Van Kempen et al., 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). All samples were analysed in triplicate (maximum standard deviation of 7% glucose release per time point).
Although multiple equations can be used to model *in vitro* starch digestion, a relative simple model was chosen to enable further statistical analysis. This resulted in the use of a modified version of the Chapman-Richards model as previously described by van Kempen et al.[35]:

Equation 2.1. $$starch\ hydrolysis = plateau * (1 - \exp(-\frac{K/100}{Plateau} * 100 * time))$$

The starch hydrolysis is expressed as % of starch in sample, plateau is the maximum amount of glucose released during digestion, which is converted to starch by multiplying with factor 0.9 (as % of sample weight) and K is the rate of glucose release corrected for plateau effects (as % of starch hydrolysed to glucose per minute). The model was fitted to the data using the average of at least triplicate observations. The K and plateau value of each starch sample was estimated by nonlinear regression procedures (proc NLIN, SAS, version 9.3, SAS Institute, Cary, USA). For estimation of the plateau value, a boundary was included forcing the estimation to be ≤100%. The model was also fitted after the plateau value was fixed to 100%, but the fit of this model was less good, as for some starches the estimated plateau value was far below 100%.

Statistical methods

Correlation coefficients between measured starch properties were generated using Pearson's correlation procedure (proc CORR) in SAS. Correlations with $P \leq 0.05$ were taken as statistically significant and $0.05 < P \leq 0.1$ as a tendency for significance.
Principal Component Analysis (PCA) was conducted using the factor procedure in SAS on the starch characteristics, to examine whether variation in these starch characteristics could be summarized in the estimation of principle components (PC's) that are uncorrelated. After extraction, PC's were scaled by their standard deviations (square roots of associated Eigenvalues) and subjected to orthogonal rotation (varimax) to obtain independent factors. The amount of variation in starch digestion kinetics explained by each independent PC was analysed with Pearson's correlation procedure.

RESULTS

Characterization of starch

To make a comparison between starches from different botanic sources and their effect on *in vitro* starch digestion kinetics, 20 starches from different botanic sources (potato, pea, corn, rice, barley, and wheat) were analysed on several characteristics regarding the molecular and granular structure of the starches.

The average granular diameter of the starches used in this study ranged from 6 to 92 µm (**Table 2.1**). In general, rice starch had the smallest granules, followed by cereal starches (of which corn had the smallest granules and wheat the largest) and pea starch, whereas potato starch consisted of the largest granules (**Figure 2.1**). As shown, barley, wheat, pea A, and potato starch had a bimodal granule distribution that consisted of a small fraction of granules with a diameter between 1 and 7 µm and a much larger fraction of granules with a diameter >7 µm. Such a bimodal distribution was also seen for waxy potato, HMT potato, corn B, high amylose corn A, high amylose corn B, and pea B starch, but not for the three rice starches, corn A, and waxy corn starch (results not shown). Granule size distribution showed that sieving of potato starch successfully separated smaller granules from larger ones (size distribution not shown, average size in **Table 2.1**).

Starch morphology was visualized by scanning electron microscopy (SEM), illustrating both variation in granular size, size distribution, and shape (**Figure 2.2**). All varieties of potato and

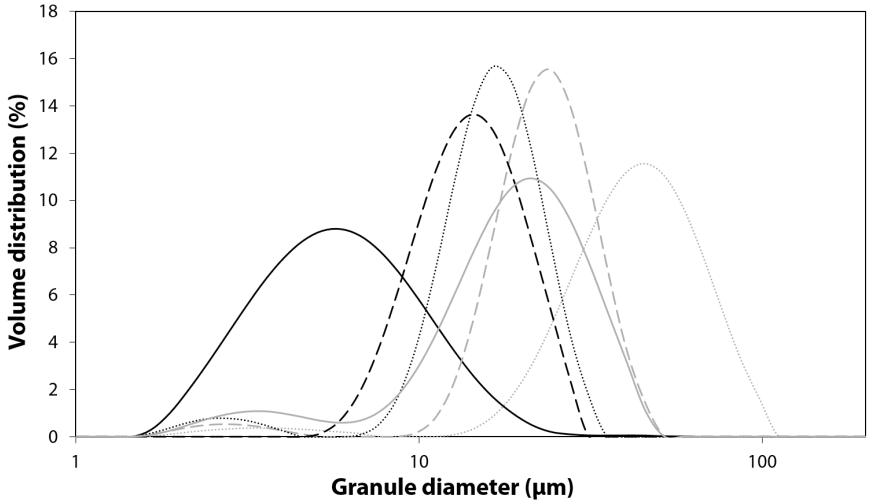

Figure 2.1. Granule diameter distribution of rice B (solid black line), corn A (dashed black line), barley (dotted black line), wheat (solid grey line), pea A (dashed grey line), and potato (dotted grey line) starch.

Table 2.1. Intrinsic properties of all analysed starch samples.

Origin	Sample	Amylose (%)	Diameter (µm)	Crystallinty (%)	A-type crystals (%)	# of pores / 100 µm²	St.dev. # of pores	mol % side-chain length (DP) amylopectin					
								Mean	6 – 12	13 – 24	25 – 36	>36	Ratio[1]
Potato	Regular	18	45.8	30.0	0.0	0.1	0.3	31	4.6	22.1	14.1	59.2	0.45
Potato	Sieved (<20)	15	18.9	30.5	0.0	0.0	0.0	31	4.3	22.4	14.6	58.8	0.45
Potato	Sieved (21-32)	18	30.8	31.5	0.0	0.0	0.0	31	4.3	22.2	14.3	59.2	0.45
Potato	Sieved (33-52)	18	49.7	31.2	0.0	0.0	0.0	31	4.6	22.5	13.8	59.1	0.46
Potato	Sieved (53-71)	18	71.2	30.6	0.0	0.2	0.4	31	4.7	22.8	13.9	58.7	0.47
Potato	Sieved (72-109)	18	92.2	22.7	0.0	0.2	0.4	29	5.3	25.1	13.9	55.7	0.55
Potato	Waxy	0	45.4	33.0	20.2	0.0	0.0	30	5.2	22.7	14.8	57.3	0.49
Potato	HMT	11	46.7	23.4	42.4	0.0	0.0	31	4.7	22.3	15.0	58.1	0.46
Pea	Regular A	27	24.7	27.3	73.4	0.0	0.0	26	6.8	29.7	20.2	43.2	0.85
Pea	Regular B	27	25.0	25.2	68.4	0.0	0.0	26	6.4	30.9	18.9	43.8	0.85
Corn	Waxy	0	16.0	28.2	100.0	36.7	25.2	23	9.6	35.3	17.9	37.3	1.20
Corn	Regular A	19	15.0	21.5	100.0	28.6	24.9	23	9.8	35.4	17.1	37.6	1.20
Corn	Regular B	19	14.6	28.2	100.0	28.6	41.9	23	10.3	36.1	17.2	36.4	1.28
Corn	High amylose A	46	13.8	28.0	41.0	3.5	8.7	32	4.5	21.5	13.6	60.4	0.43
Corn	High amylose B	55	11.0	33.7	41.3	3.3	9.9	33	3.9	18.8	13.8	63.5	0.36
Barley	Regular	20	17.3	28.3	100.0	1.1	2.0	23	7.7	29.1	20.5	42.7	0.86
Wheat	Regular	23	20.6	25.5	100.0	0.0	0.0	23	9.9	31.2	18.8	40.2	1.02
Rice	Waxy	1	8.3	27.1	100.0	13.0	26.0	23	11.1	33.2	15.1	40.6	1.09
Rice	Regular A	16	6.6	24.2	100.0	21.7	72.7	24	9.3	35.1	14.2	41.4	1.07
Rice	Regular B	14	6.2	28.5	100.0	10.8	29.3	26	7.7	27.3	17.4	47.7	0.73

[1] Ratio between short to long side chains. "Short" refers to amylopectin side-chains with DP 6-24 and "long" refers to amylopectin side-chains with DP>36

pea starch granules were large and round, whereas pea starch was more bean-shaped. All corn starches contained a mixture of squared and round granules, whereas barley starch had more disk-shaped granules. Wheat starch granules were roundly shaped and rice starch contained small, square shaped granules. Some starches seemed to stick together, which was especially clear for wheat and rice starches. The variation identified in granule size with SEM was comparable with the measured granule size distribution.

Starch surface and presence of pores was studied with SEM, visualising that all varieties of potato and pea starch had granules with a smooth surface without pores (**Figure 2.3**). The smoothness of the surface of corn starch depended on the type of corn starch. Regular corn starches (corn A and B) had smooth granules, with small, round pores. Waxy and high amylose corn starches had granule surfaces that were less smooth, more porous and granules had more and irregular shaped pores. Barley starch had a smooth surface without pores, but merely scratches on several granules. Wheat starch had the smoothest surface of all starches, with no pores, cavities or irregularities. All varieties of rice starch had a smooth surface, with several small and round pores. The average number of pores (expressed per 100 μm^2 of surface area, **Table 2.1**) enables quantitative comparison amongst samples. Most pores were observed in corn and rice samples, whereas starch from potato, pea, barley, and wheat all had none or a very limited number of pores.

Crystalline structure was studied with XRD. X-ray diffraction patterns of several starch samples are shown (**Figure 2.4**) with arrows indicating peaks that are typical for A-type or B-type

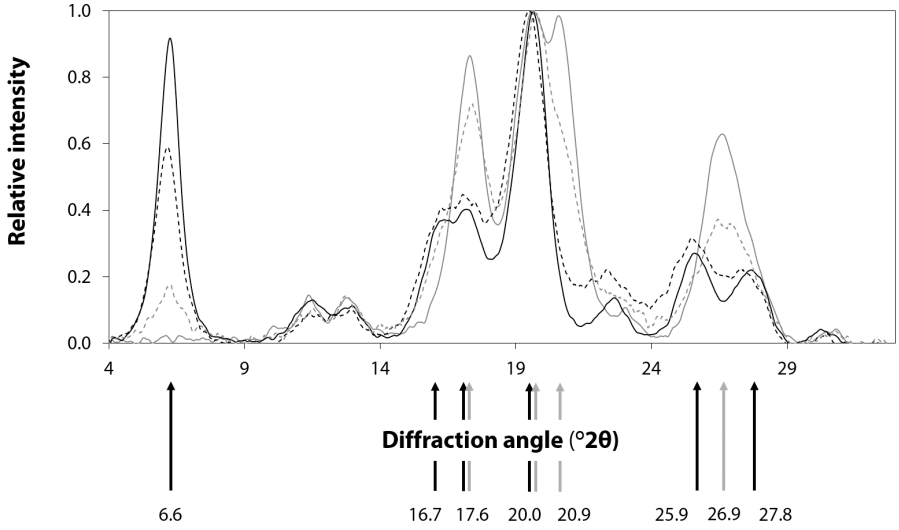

Figure 2.4. Wide-angle X-ray diffraction patterns for a typical type B crystalline starch (potato, solid black line), a typical type A crystalline starch (waxy rice, solid grey line), a typical type Ca crystalline starch (pea B, dashed grey line) and a typical type Cb crystalline starch (waxy potato, dashed black line). Arrows indicate peaks that are typical for B-crystalline starch (black) or for A-crystalline starch (grey), below which the corresponding diffraction angles are shown.

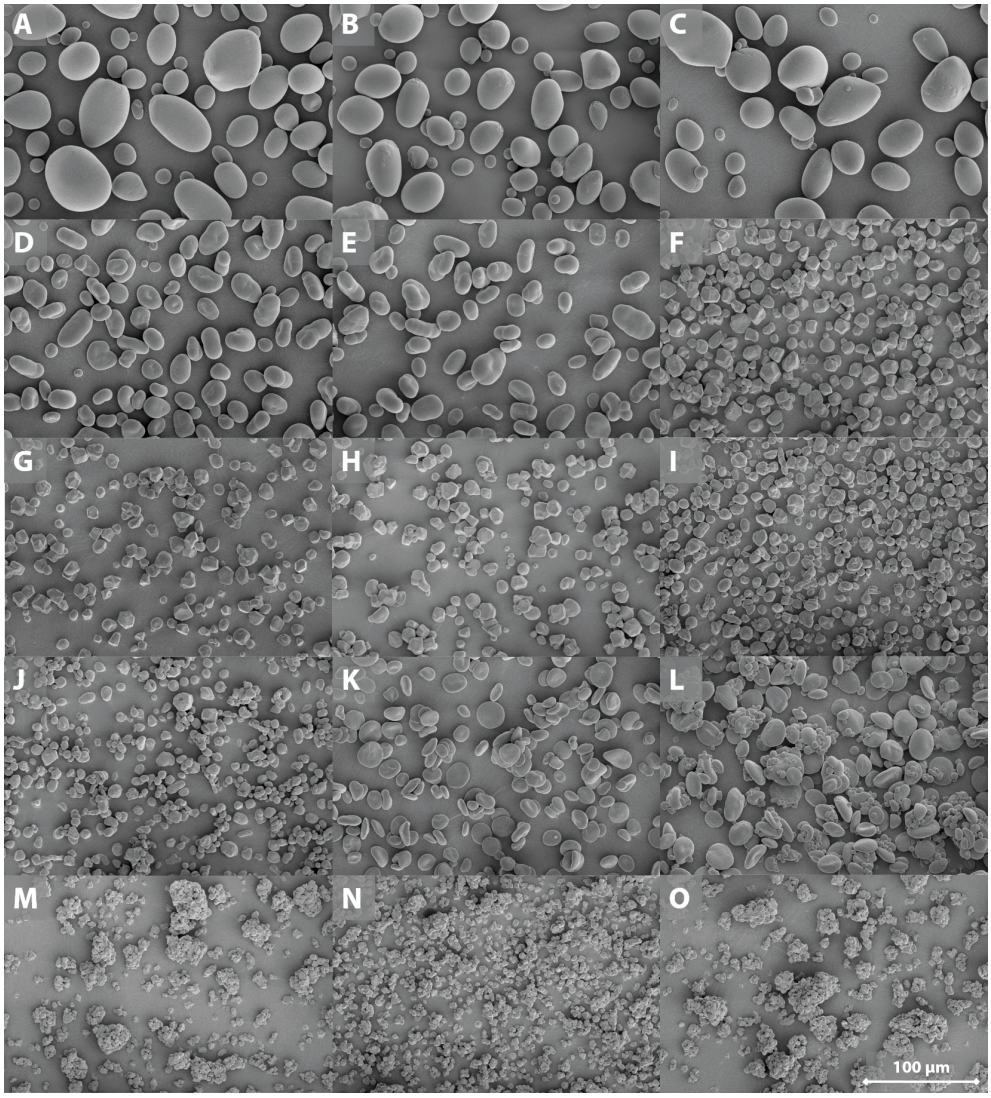

Figure 2.2. SEM pictures of the morphology of all starch samples (magnitude 1000 times). A = regular potato, B= waxy potato, C = HMT potato, D = pea A, E = pea B, F = waxy corn, G = corn A, H = corn B, I = high amylose corn A, J = high amylose corn B, K = barley, L = wheat, M = waxy rice, N = rice A, O = rice B.

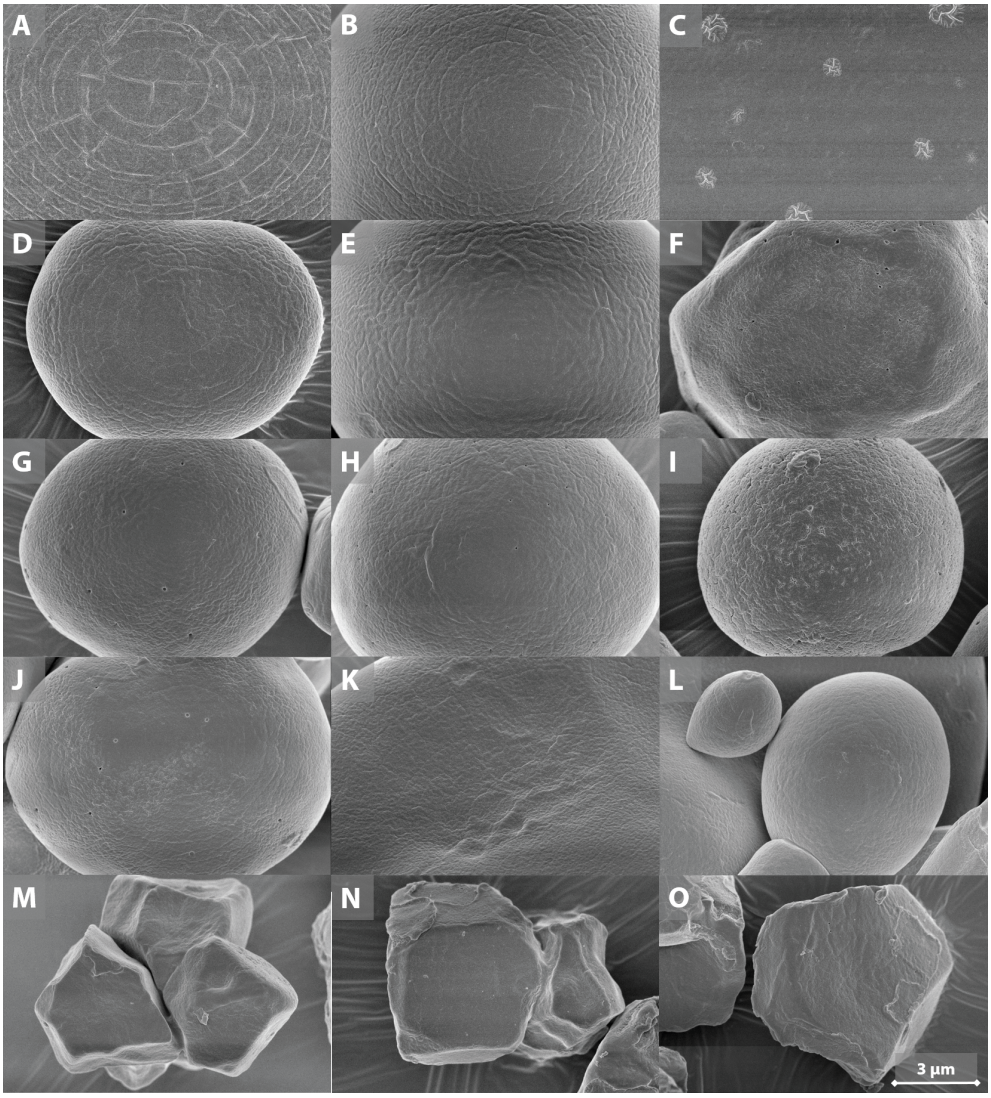

Figure 2.3. SEM pictures of the surface of all starch samples (magnitude 25000 times). A = regular potato, B= waxy potato, C = HMT potato, D = pea A, E = pea B, F = waxy corn, G = corn A, H = corn B, I = high amylose corn A, J = high amylose corn B, K = barley, L = wheat, M = waxy rice, N = rice A, O = rice B.

crystalline starch[36]. X-ray diffraction patterns of waxy corn, corn A, corn B, barley, wheat, waxy rice, rice A, and rice B starches contained solemnly representative peaks of A-type crystals and were therefore characterized as 100% A-type crystalline starch (**Table 2.1**). Potato starch contained only representative peaks for B-type crystalline starch. Some starch samples contained representative peaks of both types of crystallinity in different intensities resulting in the so-called C-type crystalline starch. This type of crystallinity can be close to B-type crystalline starch (type Cb) as observed for waxy potato, HMT potato, and high amylose corn, or closer to A-type crystalline starch (type Ca), as observed for both pea starches. Diffraction patterns of a typical B-type crystalline starch (potato) and a typical A-type crystalline starch (waxy rice) were used to calculate the exact proportion of A-type crystallites in each starch sample (**Table 2.1**).

The relative amylose content of the starches ranged from 0% for waxy samples (waxy rice, waxy corn, and waxy potato) to 55% for high amylose corn starch (**Table 2.1**).

Amylopectin side-chain length distribution ranged generally from short side-chains identified in cereal starches to long side-chains for potato starches; pea starches had an intermediate side-chain length distribution (**Figure 2.5**). Concentrations of individual branches could be calculated up to DP 42, as chains with a larger DP could not be separated with the method used. Therefore, concentration of chains larger than DP 42 are presented as the sum of all soluble chains with DP>42.

In vitro digestibility

Digestion kinetics of all starches during incubation with porcine pancreatin, amyloglucosidase and invertase was measured over time and used to fit a first order kinetics model (**Table 2.2**

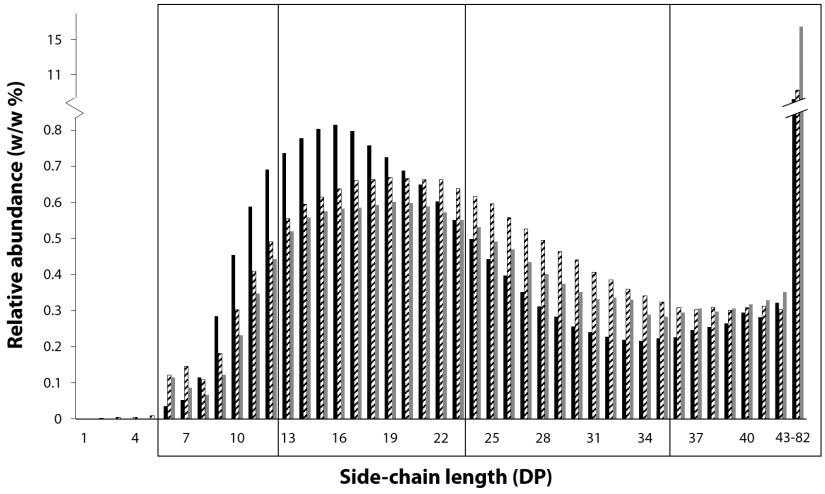

Figure 2.5. Amylopectin side-chain length distribution of rice A (black) , pea A (dashed) and potato (grey) starch. Clusters of side-chains are indicated with black boxes.

and Supplementary information **Table S2.1**). The average glucose recovery per time point was used to fit the model. *In vitro* cumulative starch hydrolysis and the estimated model are illustrated (**Figure 2.6**) for wheat being the most rapidly digestible starch tested, for potato as most resistant starch and for three intermediate digestible starch samples; corn, high amylose corn, and pea. The K value ranged from 0.08 %/min for the more resistant types of starch, to 4.19 %/min for rapidly digestible types of starch. Using data up to 360 min of digestion, the plateau value was estimated at >95% for most starch samples except for waxy potato (40%), both high amylose corn starches (63%, 67%), and pea A starch (93%).

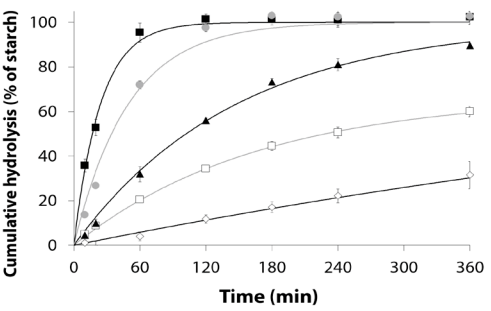

Figure 2.6. *In vitro* digestion kinetics for cereal starch (wheat ■, corn B ●, and high amylose corn B □), legume starch (pea B ▲), and tuber starch (potato ◇). Symbols indicate the average of in triplicate measured values, lines represent the first-order kinetic model fitted to these data.

Interrelationships among starch properties and digestion kinetics

Prior to correlating the structural starch properties with *in vitro* digestion kinetics, interrelations among the measured starch properties were examined and illustrated with PCA (**Table 2.3**). The first Principal Component (PC1), explaining 55% of the variance in starch properties, consisted of high loadings of 4 of the 6 parameters, namely granule diameter (-0.85), pores (0.74), type of crystalline structure (0.93), and side-chain length of amylopectin (0.86), illustrating the interrelationships of these parameters among the analysed starch sources. The amylose content was the only parameter with a high loading (0.91) on PC2, explaining 22% of the variation. The proportion of crystalline structure is loaded comparably strong, but in opposite directions, on PC1 and PC2 (-0.48 vs 0.41 respectively).

The potential of PC1, PC2 and each of the individual starch properties to explain variation in the rate of starch degradation is presented in **Table 2.4**. Of the two PC's, only PC1 has a significant correlation with the rate of *in vitro* starch digestion ($r = 0.67$, $P = 0.0061$). This indicates that PC1, combining granule diameter, pores, type of crystalline structure, and the side-chain length distribution of amylopectin, is associated with variation in starch digestion rate. This association is less strong for PC2, mainly reflecting the effect of the amylose content. As the proportion of crystalline structure is loaded on both PC1 and PC2 equally, but with loadings below 0.5, this property appears to explain little variation in digestion kinetics.

Although 4 of 6 parameters have high loadings on PC1, the combination of parameters unexpectedly does not explain more variation in K ($r = 0.67$, $P = 0.0061$) than some of the parameters individually, such as the type of crystalline structure ($r = 0.81$, $P = 0.0003$) and the fraction of short (DP 6-12) amylopectin side-chains ($r = 0.81$, $P = 0.0002$).

Table 2.2. K and plateau values for all analysed starch sources, as estimated with Equation 1.

Origin	Sample	K (%/min)	Plateau (%)[1]	Residual sum of squares
Potato	Regular	0.10	100.0	11.8
Potato	Sieved (<20)	0.14	100.0	7.9
Potato	Sieved (21-32)	0.10	100.0	7.6
Potato	Sieved (33-52)	0.08	100.0	31.7
Potato	Sieved (53-71)	0.08	100.0	2.3
Potato	Sieved (>71)	0.09	100.0	1.4
Potato	Waxy	0.13	41.4	1.1
Potato	HMT	0.41	100.0	11.3
Pea	Regular A	0.67	92.7	74.2
Pea	Regular B	0.68	99.9	24.7
Corn	Waxy	2.55	100.0	44.7
Corn	Regular A	1.89	100.0	197.5
Corn	Regular B	2.03	100.0	168.7
Corn	High amylose A	0.41	63.1	14.5
Corn	High amylose B	0.40	67.3	6.9
Barley	Regular	3.22	100.0	95.5
Wheat	Regular	4.15	100.0	47.7
Rice	Waxy	4.29	100.0	5.6
Rice	Regular A	1.60	95.5	27.0
Rice	Regular B	3.06	95.6	69.0

[1]Maximised to 100%.

Discussion

Previous research has focussed on the contribution of starch digestion to dietary energy supply, but currently also the effects of variation in starch digestion kinetics have become more clear[1-4]. Properties related to the molecular and granular structure of starch cause variation in starch digestion kinetics[13,14,16,21]. However, quantifying the contribution of every unique property is complicated by inherent combinations of properties within starches originating from a similar botanic source[14,29-32]. Previous work showed that it is complicated to estimate the effect of each individual starch characteristic across botanic sources based on literature[33]. Therefore, this research aimed to identify, across botanic sources, the starch characteristics that are affecting starch digestion most in starches that are relevant for feed production for monogastric animals.

Starch properties

Most of the measured starch properties are close to values that were published before[6,10,11,30,37-43], except the lack of bimodal size distribution in waxy corn and corn A, which is presumable the result of differences in isolation procedures between starch wholesalers. The low number of pores observed in barley and wheat starch partly contradicts previous literature[12,44], in which more pores were observed for both cereals. This difference in pore abundance may be related to heterogeneity of the analysed starches and to differences in the varieties analysed in our study compared with previous studies. In this study, a large number of individual granules were studied from a homogenous sample, providing a reliable insight in the number of pores.

Table 2.3. Rotated factor pattern, eigenvalues and proportion variance explained of principle components in multivariate analysis.

	PC1	PC2
Eigenvalues	3.30	1.29
Proportion variance explained	0.55	0.22
Loading of variables		
Granule diameter	-0.85	-0.46
Number of pores	0.74	-0.31
Crystal content	-0.48	0.41
Percentage A-type crystals	0.93	-0.12
Amylose content	-0.10	0.91
Ratio amylopectin side-chains	0.86	-0.40

Starch digestion kinetics

The inevitable consequence of a model with only two parameters, is an imperfect fit of data for some starch sources (**Table 2.2**, **Figure 2.6**). However, it is a necessity to use the same, relatively simple model for all sources in order to relate the rate of digestion to starch properties. For some starches, the extent of starch digestion did not reach the estimated plateau value within 360 min of incubation and the plateau value was estimated at values below 100%. To obtain more insight in the biological meaning of the estimated plateau value, a prolonged incubation (t=24h) was performed with a selection of those starches, namely high amylose corn A and pea A starch. During this prolonged incubation the extent of starch digestion exceeded the estimated plateau value (84.1 and 100% respectively). This indicates that the estimated plateau value is required for a good fit of data, but does not necessarily reflect the asymptotic maximum glucose release for all starch samples, as stated by van Kempen et al.[45] and Englyst et al.[34]. Therefore the rate of starch digestion, but not the extent, was used to study correlations between digestion kinetics and starch properties.

Interrelationships among starch properties and digestion kinetics

As illustrated with PCA, variation in starch properties could be summarised in two independent factors, but neither factor additively explains variation in digestion kinetics. To understand the contribution of each distinct starch property to the variation observed in starch digestion kinetics, individual properties are further discussed in groups of starches that have similarities in their botanical origin and in groups of starches that have similar properties.

Amylose content affects starch digestion kinetics in starches from cereal origin

The observation that amylose content, a high loading on PC2, is not associated with starch digestion kinetics, contradicts with previous research[13-16]. However, these previous studies were performed with starches from cereal origin only, whereas in this study starches from other botanical origins were also included. Therefore, PCA analysis and Pearson's correlation analysis were repeated with a subdataset that contained data from cereal starches only (supplementary information, **Table S2.2** and **Table S2.3**). Those analysis revealed a significant correlation (r = -0.72, P = 0.03) between amylose content and starch digestion rate. However, within these cereal starches, interrelationships between amylose content, pores, type of crystalline structure, and the side-chain length distribution of amylopectin are present as well, which were absent in the complete dataset. Those interrelationships between starch characteristics make it impossible to separate effects of the amylose content from other starch characteristics and to draw conclusions on the role of amylose on digestion kinetics.
We conclude that an increased amylose content negatively correlates with starch digestion kinetics when comparing starches within botanic sources, but is not the largest factor in explaining variation in starch digestion kinetics across botanic sources. This is clearly illustrated by a significant lower (P<0.01) starch hydrolysis of waxy corn (36%) compared with barley (45%) and wheat starch (53%) after 20 min of *in vitro* digestion. Barley, wheat and waxy corn starch have comparable characteristics, except for the proportion of amylose and the number of pores, as waxy corn starch has a lower amylose content and more pores than barley and wheat starch. This indicates that, across botanic sources, a starch with a low amylose content is not necessarily more rapidly digestible than starches with a normal amylose content.

The number of pores does not unambiguously predict variation in starch digestion kinetics

In general, pores are believed to enable enzymes to digest starch granules from the inside out, which increases the rate of hydrolysis of the granules[12,28]. However, several starch samples with no pores or a low number of pores (barley and wheat) reached a significant (P<0.0001) higher starch hydrolysis after 20 minutes of *in vitro* digestion (45 and 53%, respectively) than corn A (25%), which has many pores (**Table 2.1**). Previous studies reporting positive correlations between pores and digestion kinetics, mainly focused on corn[12,28]. Indeed, within our corn starches, the number of pores is also positively associated with K (r = 0.94, P = 0.0173). However, correlations between the number of pores and the amylose content (r = -0.95, P = 0.0324), and number of pores and the percentage of A-type crystals (r = 0.89, P = 0.0004) are also observed within these samples and have additionally been shown for barley starch previously[46]. Therefore, we conclude that even though the number of pores is associated with other starch properties, it may causally explain variation in starch digestion kinetics within a botanic source, but not across botanic sources.

Table 2.4. Pearson correlation coefficients for starch properties and digestion kinetics, of all analysed (non-sieved) starch samples[1].

		PC1	PC2	Amylose content	Granule diameter	Crystal content	A-type crystallinity	Number of pores	Side-chain length amylopectin (DP)					
									6 - 12	13 – 24	25 – 36	> 36	Ratio[2]	K
PC1		X	0.00	-0.10	-0.85**	-0.48*	0.93**	0.74**	0.85**	0.84**	0.36	-0.82**	0.86**	0.67**
PC2			X	0.91**	-0.46	0.41	-0.12	-0.31	-0.39	-0.43*	-0.14	0.40	-0.39	-0.17
Amylose content				X	-0.24	0.18	-0.24	-0.36	-0.48*	-0.45*	-0.12	0.43	-0.42	-0.37
Granule diameter					X	0.12	-0.74**	-0.50*	-0.56**	-0.48*	-0.13	0.48*	-0.51*	-0.57**
Crystalline content						X	-0.49*	-0.31	-0.45*	-0.56**	-0.26	0.53*	-0.51*	-0.25
A-type crystallinity							X	0.57**	0.88**	0.84**	0.56**	-0.88**	0.86**	0.81**
Number of pores								X	0.65**	0.69**	-0.01	-0.59**	0.70**	0.33
Side-chain amylopectin (DP) length	**6 - 12**								X	0.93**	0.40	-0.92**	0.95**	0.81**
	13 - 24									X	0.49*	-0.98**	0.99**	0.60**
	25 - 36										X	-0.65**	0.49*	0.42
	> 36											X	-0.98**	-0.68**
	Ratio												X	0.65**
K														X

[1] ** indicates a significant correlation (P≤0.05), * indicates a tendency for a significant correlation (0.05< P ≤0.10).

[2] Ratio between short to long side chains. "Short" refers to amylopectin side-chains with DP 6-24 and "long" refers to amylopectin side-chains with DP>36.

Granule diameter alone does not predict variation in starch digestion kinetics

A significant correlation between the starch granule diameter and k value was identified (r = -0.57, P = 0.03), when analysing the complete set of starches. However, it is not clear whether this correlation is the result of collinearity between the granule diameter and another starch parameter, or whether the granular size is affecting the digestion rate. Consequently, additional statistical analysis of sieved fractions of potato starch provides was performed. Those five sieved starch fractions differ primarily in average granule diameters, varying from 18.9 µm to 92.2 µm, whereas the difference among the other properties was negligible (**Table 2.1**). Within these sieved fractions, granule diameter was not significantly correlated with K (r = -0.65, P = 0.2307), demonstrating that for the potato starch used in this study, granule diameter, and consequently surface area, has no causal relation with digestion kinetics. Previous research on the relation between granule diameter and digestion kinetics showed interrelationships between variables such as granule diameter and amylose content[26,47] and the ability of α-amylase to adhere to granules[48]. This implies that granule diameter and surface area are not causally related to starch digestion kinetics.

The type of crystalline structure and chain length distribution of amylopectin explain variation in digestion kinetics across botanic sources

After eliminating the granule diameter and number of pores as causal factors affecting starch digestion kinetics across botanic sources, only the percentage of A-type crystals and ratio of short (DP<24) to long (DP>36) amylopectin side-chains remain as starch characteristics that affect starch digestion kinetics. Both show strong significant positive correlations with K (r = 0.81, P = 0.0003; r = 0.65, P = 0.0081 respectively) but they also have a strong correlation with each other (**Figure 2.7**), confirming previous data[14,20-23,40,42]. In general, low and normal amylose cereal

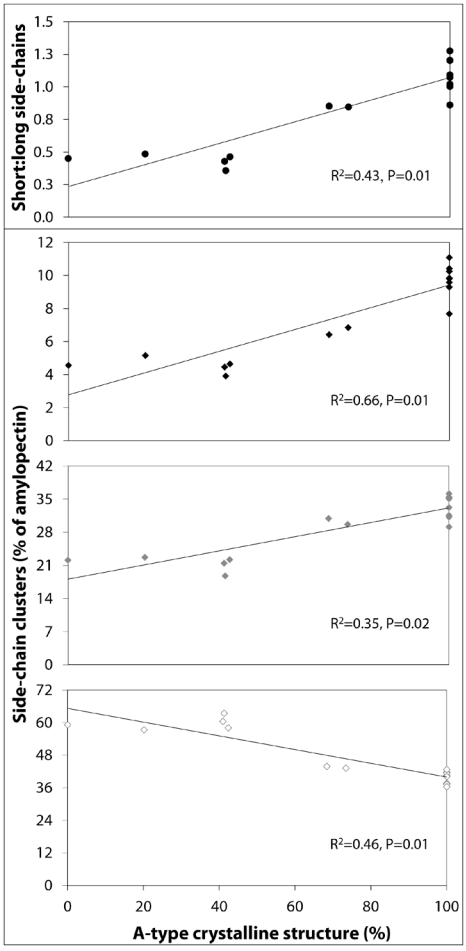

Figure 2.7. Relation between type of crystalline structure and ratio of short:long amylopectin side-chains (●) and side-chain length clusters DP 6 – 12 (◆), DP 13 – 24 (◇), and DP > 36 (◈) within all (non-sieved) starch samples.

starches consist of both A-type crystals and a high ratio of short:long amylopectin side-chains, whereas potato starch displays B-type crystals and longer amylopectin side chains, and pea and high amylose cereal starches have intermediate crystalline types and amylopectin chain length distributions. We therefore conclude that these are the only measured characteristics that explain variation in digestion kinetics among botanic sources.

Characteristics that additionally explain variation in starch digestion depend on the botanic origin.

The proportion of variance (R2) in digestion kinetics explained by PC1 across all botanic sources did not exceed 45% and was lower than the type of crystalline structure alone (R2=66%). In an attempt to further challenge the concept of additivity, interrelations between the measured starch properties were examined within several clusters of botanic sources and illustrated with PCA. The structural starch properties were correlated with *in vitro* digestion kinetics within those clusters of botanic sources. When exploring the correlations between starch properties, the lack of additivity proved to be highly affected by the combination of selected botanic sources that were evaluated statistically. For example, multivariate analysis with only corn, pea and potato starches resulted in an PC loading profile that is comparable with the one obtained from the full dataset (supplementary information, **Table S2.4**). However, instead of 45%, 93% of the variation in starch digestion kinetics within this botanic cluster can be explained with this combination of starch characteristics. The combination of characteristics also additively explains variation, as R2 for PC1 (93%) exceeds that of the type of crystalline structure (81%), the number of pores (89%), and the ratio of short:long amylopectin side chains (87%) individually (supplementary information, **Table S2.5**). Alternatively, there may be a role for other starch properties in explaining starch digestion behaviour, which were not included in this study. For example, previous studies proved that digestion kinetics can also be affected by minor components present such as proteins, lipids and phosphorus, which make up <1.5% of the starch granule[6] and by variation in amylose structure[49]. However, based on literature, we believe that the selection of starch characteristics made for this study, covers the most important variation in molecular and structural properties of the starch granule. The lack of additivity of those starch properties in relation to variation in starch digestion kinetics, indicates that variation in most starch properties explains variation within, rather than among botanic sources.

Conclusions

Across all analysed botanic sources, the type of crystalline structure and the amylopectin side-chain length distribution predicts most variation in *in vitro* digestibility kinetics among starches commonly used in pig nutrition. Granule size is not causally related to starch digestion kinetics, and amylose content and number of pores appeared to explain variation within rather than across botanical sources. Furthermore, within (clusters of) botanical sources variation in digestion kinetics is additively explained by other starch properties measured.

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Table S2.1. *In vitro* digestion of all analysed starches.

Origin	Sample	Average cumulative starch hydrolysis ± standard deviation (%)							
		0 min	10 min	20 min	60 min	120 min	180 min	240 min	360 min
Potato	Regular	-1±0.6	1±0.1	-1±2.2	4±2.0	12±1.9	17±2.4	22±3.1	32±6.1
Potato	Sieved (<20)	0±0.0	2±0.5	4±0.5	8±0.3	17±1.7	21±0.6	28±0.9	40±2.7
Potato	Sieved (21-32)	0±0.0	0±0.4	2±0.5	5±0.3	11±0.1	16±1.0	21±1.0	31±2.8
Potato	Sieved (33-52)	0±0.1	1±0.8	2±0.8	4±0.8	8±1.0	12±1.5	17±1.3	25±2.5
Potato	Sieved (53-71)	0±0.1	1±0.6	2±0.6	4±0.9	9±0.9	13±0.6	18±1.3	26±2.8
Potato	Sieved (72-109)	-1±1.0	0±0.8	2±0.5	5±0.2	10±0.8	15±0.9	20±0.9	28±2.3
Potato	Waxy	0±0.1	2±0.1	3±0.2	8±0.2	13±0.9	18±0.5	22±1.2	28±0.6
Potato	HMT	0±0.1	3±1.2	6±0.3	21±1.0	39±1.2	53±1.6	63±1.5	78±0.7
Pea	Regular A	0±0.7	4±3.9	10±3.9	33±4.9	55±5.2	69±5.5	75±6.6	86±7.3
Pea	Regular B	0±0.1	5±2.2	10±1.9	32±3.5	56±1.1	73±1.5	81±2.7	90±1.3
Corn	Waxy	-1±0.6	21±1.7	36±3	81±2.0	98±1.3	102±5.3	100±5.3	99±7.2
Corn	Regular A	0±0.1	11±3.2	25±1.3	70±3.6	95±4.5	101±1.2	103±1.6	106±3.2
Corn	Regular B	0±0.3	14±1.1	27±1.1	72±1.9	98±1.7	103±2.1	102±4.0	103±2.3
Corn	High amylose A	0±0.6	6±1.3	6±4.6	21±3.1	34±3.5	44±1.5	50±3.7	57±4.9
Corn	High amylose B	0±0.4	5±0.5	9±0.5	20±1.3	34±0.9	45±2.1	51±2.5	60±2.3
Barley	Regular	0±0.2	22±2.6	45±3.1	93±5.6	100±3.1	100±4.9	101±4.2	100±2.6
Wheat	Regular	0±0.3	34±2.4	57±2.5	94±0.5	99±1.0	101±3.2	101±3.4	100±1.1
Rice	Waxy	0±0.3	36±2.9	53±3.6	95±4.2	101±2.3	102±2.9	101±3.3	102±3.5
Rice	Regular A	0±0.2	32±2.5	42±1.5	81±2.5	95±2.6	94±3.0	95±2.5	96±0.7
Rice	Regular B	0±0.5	18±3.8	27±2.7	61±3.4	81±2.5	90±4.7	94±3.0	96±3.2

Table S2.2. Rotated factor pattern, eigenvalues and proportion variance explained of principle components in multivariate analysis of subdataset 1[1].

	PC1	**PC2**
Eigenvalues	3.58	1.11
Proportion variance explained	0.60	0.19
Loading of variables		
Granule diameter	0.03	0.97
Number of pores	0.72	-0.28
Crystal content	-0.71	-0.22
Percentage A-type crystals	0.91	-0.07
Amylose content	-0.91	0.16
Ratio short:long amylopectin side-chains	0.95	0.16

[1] Subdataset 1 contains only data from cereal starches.

Table S2.3. Pearson correlation coefficients for starch properties and digestion kinetics, within subdataset 1[1,2].

		PC1	PC2	Amylose content	Granule diameter	Crystal content	A-type crystallinity	Number of pores	Side-chain length amylopectin, DP					
									6 – 12	13 – 24	25 – 36	>36	Short:long[3]	K
PC1		X	0.00	-0.91**	-0.03	-0.71**	0.91**	0.72**	0.93**	0.97**	0.43	-0.95**	0.95**	0.56*
PC2			X	0.16	0.97**	-0.22	-0.07	-0.28	-0.10	0.08	0.58**	-0.22	0.046	-0.21
Amylose content				X	0.09	0.46	-0.88**	-0.59*	-0.87**	-0.83**	-0.44	0.85**	-0.82**	-0.72**
Granule diameter					X	-0.12	-0.06	-0.13	-0.09	0.10	0.60*	-0.23	0.19	-0.17
Crystalline content						X	-0.58*	-0.41	-0.63*	-0.69**	-0.19	0.64**	-0.64**	-0.27
A-type crystallinity							X	0.43	0.90**	0.87**	0.65**	-0.94**	0.86**	0.75**
Number of pores								X	0.51	0.68**	0.00	-0.56*	0.66**	-0.06
Side-chain length amylopectin (DP)	**6 - 12**								X	0.94**	0.42	-0.95**	0.95**	0.71**
	13 - 24									X	0.40	-0.97**	0.99**	0.49
	25 - 36										X	-0.60*	0.45	0.63*
	> 36											X	-0.98**	-0.64**
	Short:long												X	0.52*
K														X

[1] Subdataset 1 contains only data from cereal starches.

[2] ** indicates a significant correlation ($P \leq 0.05$), * indicates a tendency for a significant correlation ($0.05 < P \leq 0.10$).

[3] "Short" refers to amylopectin side-chains with DP 6-24 and "long" refers to amylopectin side-chains with DP>36.

Table S2.4. Rotated factor pattern, eigenvalues and proportion variance explained by principle components in multivariate analysis of subdataset 2[1].

	PC1	PC2
Eigenvalues	3.18	1.45
Proportion variance explained	0.53	0.24
Loading of variables		
Granule diameter	-0.62	-0.69
Number of pores	0.88	-0.14
Crystal content	-0.45	0.51
Percentage A-type crystals	0.96	0.09
Amylose content	-0.10	0.80
Ratio short:long amylopectin side-chains	0.94	-0.21

[1] Subdataset 2 contains only data from corn, pea and potato starches.

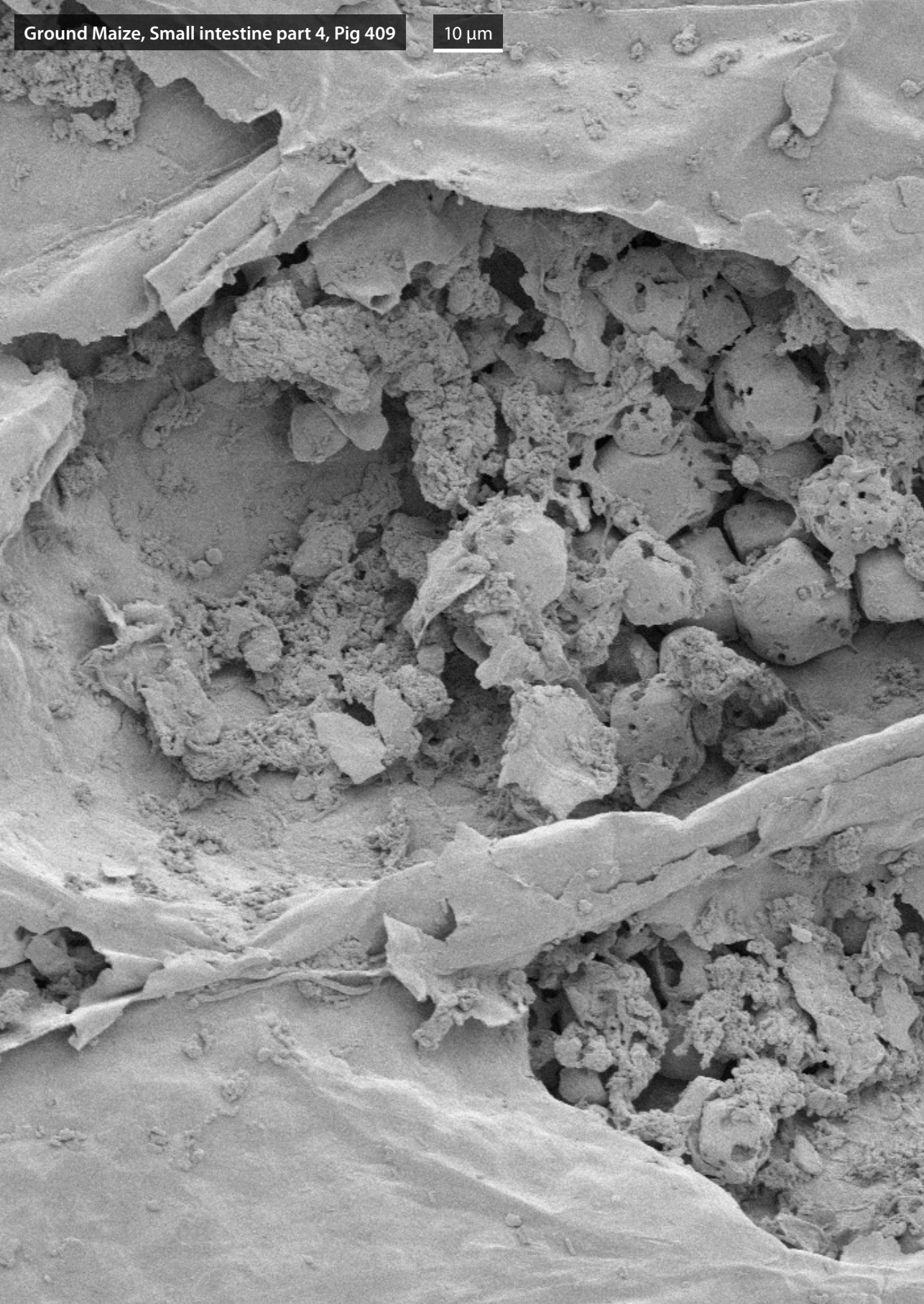
Table S2.5. Pearson correlation coefficients for starch properties and digestion kinetics, within subdataset 2[1,2].

		PC1	PC2	Amylose content	Granule diameter	Crystal content	A-type crystallinity	Number of pores	Side-chain length amylopectin, DP					
									6 – 12	13 – 24	25 – 36	> 36	Short:long[3]	K
PC1		X	0.00	-0.10	-0.62**	-0.45*	0.96**	0.88**	0.93**	0.90**	0.72*	-0.91*	0.94**	0.97**
PC2			X	0.80**	-0.69**	0.51*	0.09	-0.14	-0.24	-0.29	-0.07	0.24	-0.21	-0.08
Amylose content				X	-0.32	0.11	0.02	-0.26	-0.30	-0.31	-0.14	0.28	-0.27	-0.21
Granule diameter					X	-0.14	-0.63**	-0.44*	-0.38	-0.34	-0.42	0.38	-0.41	-0.53**
Crystalline content						X	-0.43	-0.30	-0.45*	-0.52**	-0.39	0.50*	-0.46*	-0.35
A-type crystallinity							X	0.76**	0.87**	0.85**	0.78**	-0.88**	0.88**	0.90**
Number of pores								X	0.85**	0.77**	0.41	-0.74**	0.82**	0.94**
Side-chain length amylopectin (DP)	**6 - 12**								X	0.97**	0.70**	-0.96**	0.99**	0.94**
	13 - 24									X	0.80**	-0.99**	0.99**	0.89**
	25 - 36										X	-0.86**	0.77**	0.62**
	> 36											X	-0.99**	-0.88**
	Short:long												X	0.93**
K														X

[1] Subdataset 2 contains only data from corn, pea and potato starches.

[2] ** indicates a significant correlation ($P \leq 0.05$), * indicates a tendency for a significant correlation ($0.05 < P \leq 0.10$).

[3] "Short" refers to amylopectin side-chains with DP 6-24 and "long" refers to amylopectin side-chains with DP>36.


Ground Maize, Small intestine part 4, Pig 409
10 µm

Chapter 3

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

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Abstract

This study aimed to examine *in vivo* 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 prediction 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 gastrointestinal 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 slowly 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 increased the rate of *in vivo* starch digestion and that an increase in amylose content decreased 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 (EA) 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 a 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 in such a way 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 one.

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 euthanised. Pigs were euthanised 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 3.1**). 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 (Lestrem, 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. Chrome (Cr) and Cobalt (Co) were included as markers in the feed at a level of 170 mg/kg (w/w, as fed basis), in the form of chromium oxide (Cr2O3) and Co-EDTA, respectively.

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

Table 3.1. Ingredient 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

[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 (EA) form.

[2] Altia corporation, Koskenkorva

[3] Roquette, Lestrem, 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

Table 3.1 (continued). Nutrient composition of diets containing barley, maize, or high amylose maize starch, included as isolated powder, ground cereal, or extruded cereal[1].

Analysed composition (DM basis)	IB	GB	EB	IC	GC	EC	IA	GA	EA
Starch (*g/kg*)	423	444	470	423	472	482	401	467	474
Amylose (% 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

[6] Calculated based on data from Centraal Veevoeder Bureau[21].

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 product 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 polymerisation (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 (A7095) and invertase (I4504), 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 3.1**)[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 the following equation:

Equation 3.1.

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

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 retention time (CRT) of starch per segment (n) of the small intestine according to the following equation:

Equation 3.2. $$CRT(n) = S * \left(MRTl(n-1) + 0.5 * MRTl(n)\right) + (1-S) * (MRTs(n-1) + 0.5 * MRTs(n))$$

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 3.3. $$starch\ hydrolysis = plateau * (1 - \exp(-\frac{K/100}{Plateau} * 100 * time))$$

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

Table 3.2. 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].

	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.

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 DC

The sum of glucose and all soluble α (1-4) maltodextrins was quantified as fraction of total unabsorbed starch residuals (supplementary information, **Table S3.1**) and used to calculate the DC of starch (**Table 3.2**) 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}, supplementary information, **Table S3.2**).

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.

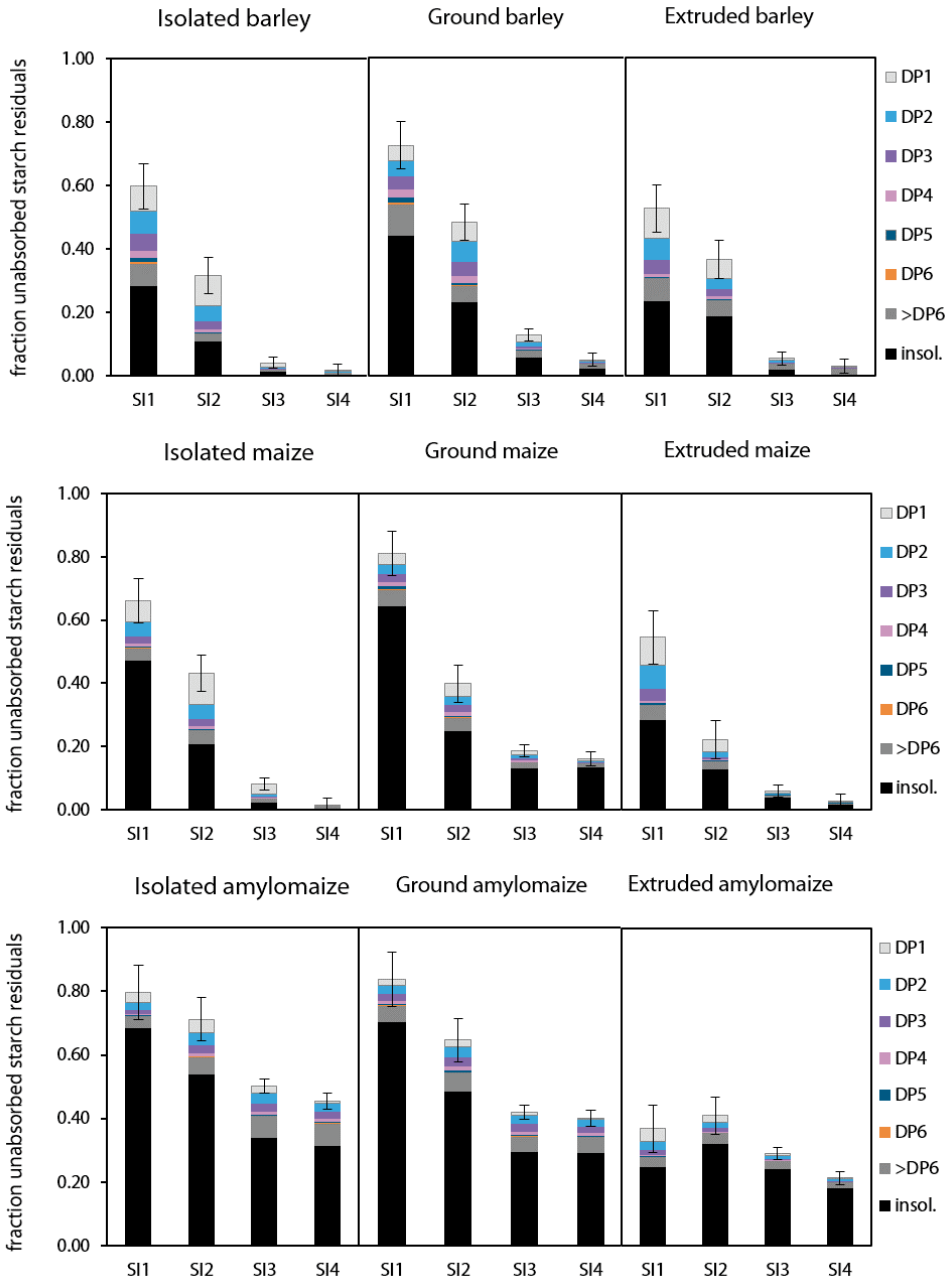

Figure 3.1. Fraction unabsorbed starch residuals, calculated as 1 - digestion coefficient (DC) in digesta recovered from 4 parts of the small intestine of pigs fed barley, maize or high amylose (HA) maize based diets which included starch as isolated powder, ground cereal or extruded cereal. Undigested starch is divided into soluble oligomers, quantified per individual oligomer up to degree of polymerisation (DP) 6, and insoluble starch. The error bars represent the standard error of the estimated mean DC.

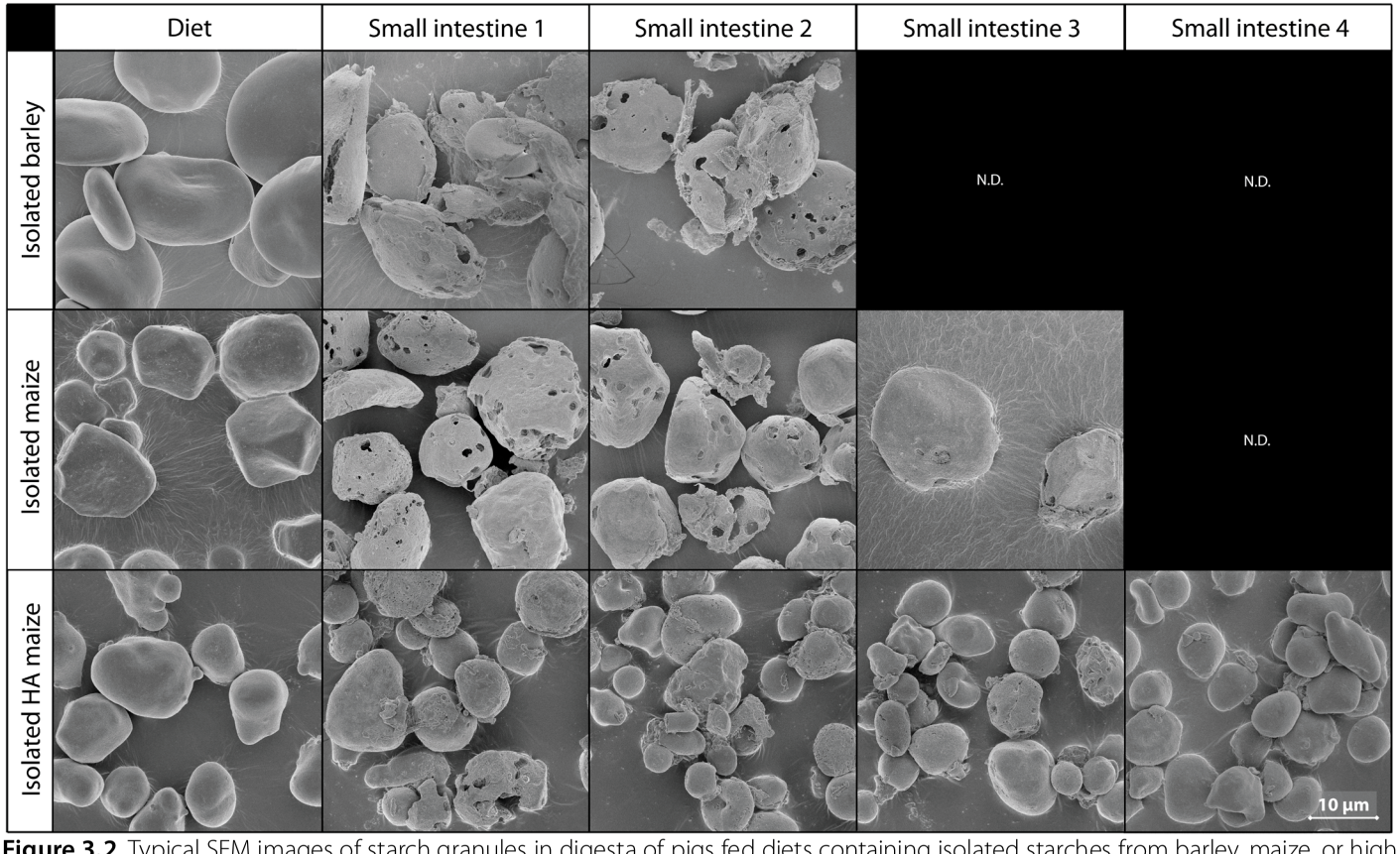

Figure 3.2. Typical SEM images of starch granules in digesta of pigs fed diets containing isolated starches from barley, maize, or high amylose maize origin, in diets and 4 segments of the small intestine, 5000x magnified. Not determined (N.D.) is used to indicate that not enough insoluble starch residues were present in those SI compartments to enable SEM analysis.

Glucose and maltodextrins release during starch digestion

A typical HPAEC elution pattern of the soluble fraction of starch residuals in small intestinal digesta (SI1-SI4) of pigs fed ground maize (supplementary information, **Figure S3.1**) 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 3.1**). 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 3.2**, 5000x magnified) and within the ground cereal matrix (**Figure 3.3**, 1000x magnified). Furthermore, starch in all ground cereals was mainly present inside a protein and cell wall matrix, which was damaged upon extrusion (**Figure 3.3**). 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 3.2**). 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 3.4** and **Figure S3.2**, supplementary information).

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 3.3**). 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

Table 3.3. *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[2].

	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 (%)	100.0	98.7	99.8	97.8	99.1	100.0	67.2	75.7	88.8
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].
[2] Starch forms are abbreviated as follows: isolated (I), ground (G), and extruded (E) form.

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

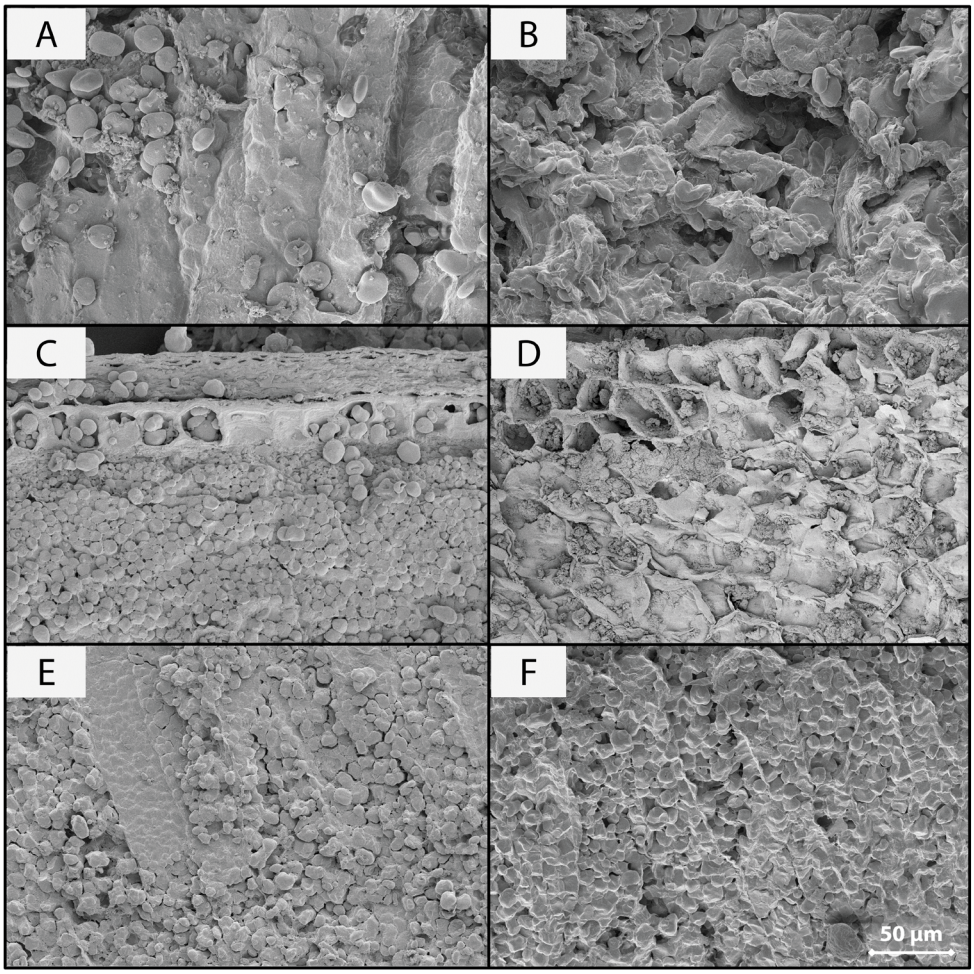

Figure 3.3. SEM images of diets containing barley in ground (A) and extruded form (B), maize in ground (C) and extruded form (D), and high amylose maize in ground (E) and extruded form (F), 1000x magnified.

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 3.4**). This indicates the presence of substantial fractions of hard endosperm, which is typically richer in indigestible proteins[14]. In contrast, only loosely

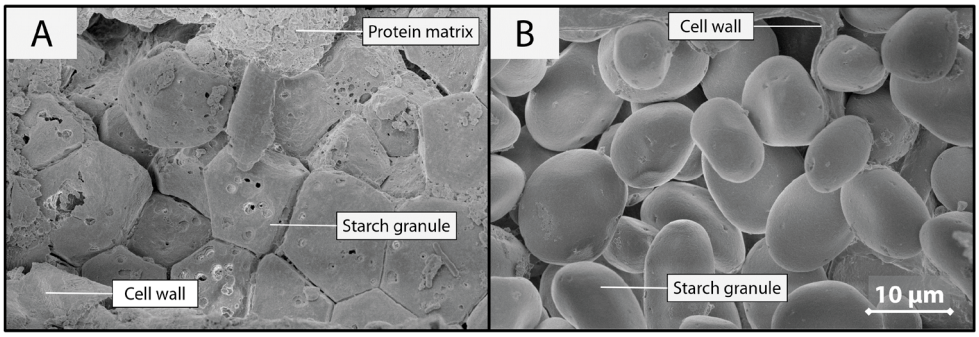

Figure 3.4. SEM image of digesta recovered from SI4 of a pig fed ground maize (A) and of a pig fed ground barley (B), 5000x magnified.

packed starch granules were identified for barley (**Figure 3.4**), 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 (supplementary information, **Figure S3.2**), 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 3.3**). 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].

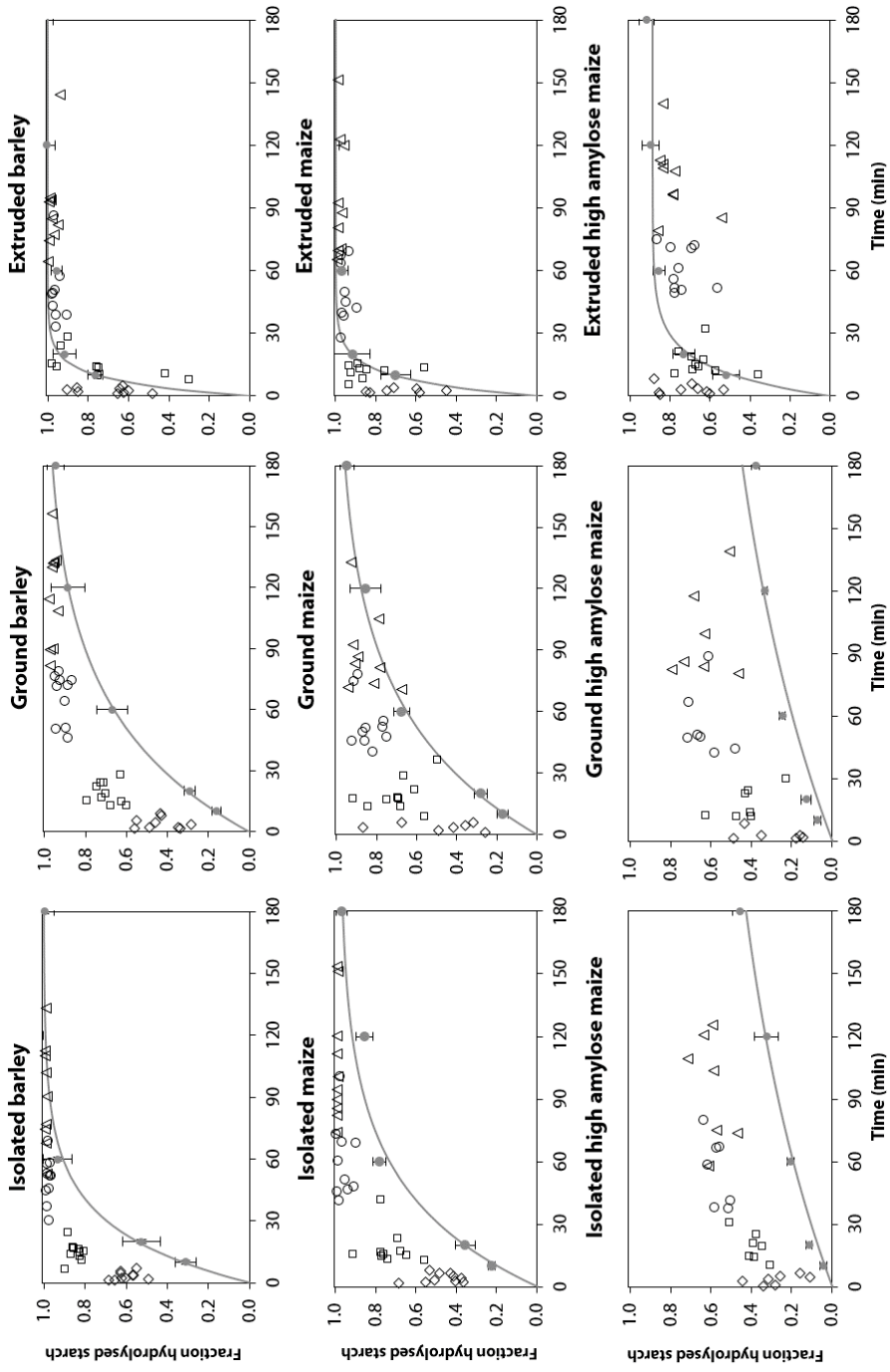

Figure 3.5. Digestion coefficients (DC) of starch measured in digesta recovered from part 1(◇), 2 (□), 3(○) and 4(△) of the small intestine of individual pigs fed barley, maize or high amylose maize based diets which included starch as isolated powder, ground cereal or extruded cereal, plotted against 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.

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 3.1**), 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 3.2**), 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 and isolated starch. In addition, the rate of *in vitro* starch digestion of ground cereals was lower than that of isolated starch. This confirms results of previous *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 3.5**), by plotting *in vivo* starch hydrolysis in all segments of all pigs on a single treatment, against the cumulative intestinal retention time (CRT, supplemental information, **Table S3.3**). 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 extent 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 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 3.1**), 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 a combination of α-amylase and brush border enzymes determine the rate of *in vivo* starch digestion, but that 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.

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Supplementary information

Table S3.1. Glucose and soluble α-(1-4) maltodextrins (% of unabsorbed starch residuals) in feed and small intestinal digesta (pooled digesta of all pigs within a treatment) of pigs fed diets containing barley, maize, or high amylose maize starch, included as isolated powder, ground cereal, or extruded cereal[1,2].

	Experimental diets								
	Barley			Maize			High amylose maize		
	Isolated	Ground	Extruded	Isolated	Ground	Extruded	Isolated	Ground	Extruded
Feed	0.9	1.5	1.0	0.9	1.1	0.9	2.1	2.0	1.9
SI1	54	40	59	31	21	51	16	18	34
SI2	73	61	54	53	40	41	25	29	23
SI3	68	62	63	76	30	36	33	34	15
SI4	69	57	100	77	16	32	30	27	15

[1] 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).
[2] Percentages of glucose and soluble α-(1-4) maltodextrins are quantified with HPAEC.

Table S3.2. Digestion coefficients calculated with the single marker method (DC_{chrome}), 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]

	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	0.39	0.34	0.53	0.34^{f}	0.21^{g}	0.53^{f}	0.21^{l}	0.18^{l}	0.65^{k}	0.21	**<0.0001**	0.441	**<0.0001**	E>I=G	
SI2	0.65	0.54	0.64	0.56^{g}	0.60^{g}	0.78^{f}	0.27^{l}	0.32^{l}	0.60^{k}	0.17	**<0.0001**	**<0.0001**	**<0.0001**	E>I=G	B=M>A
SI3	0.95^{a}	0.86^{b}	0.94^{a}	0.91^{f}	0.80^{g}	0.94^{f}	0.47^{m}	0.54^{l}	0.71^{k}	0.06	**<0.0001**	**<0.0001**	**<0.0001**	E>I>G	B>M>A
SI4	0.99	0.94	0.98	0.98^{f}	0.84^{g}	0.98^{f}	0.54^{l}	0.57^{l}	0.79^{k}	0.06	**<0.0001**	**<0.0001**	**<0.0001**	E>I>G	B>M>A
p-value source[8]	**<0.0001**			**<0.0001**			**<0.0001**								
Effect[9]	SI1<SI2<SI3<SI4			SI1<SI2<SI3<SI4			SI1=SI2<SI3<SI3								
Form	Isolated			Ground			Extruded								
p-value form	**<0.0001**			**<0.0001**			**<0.0001**								
Effect	SI1<SI2<SI3<SI4			SI1<SI2<SI3<SI4			SI1<SI2<SI3<SI4								

[1] Presented values are estimated means 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 are abbreviated as follows: isolated (I), ground (G), and extruded (E) form.

[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 found, 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 ($P<0.05$), symbol > indicates which source or form has a larger DC than others, whereas symbol = indicates that the DC between sources or forms did not differ.

[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, analyzed per source.

[9] When a segment effect is present ($P<0.05$), symbol < indicates in which segment DC was smaller than in others, whereas symbol = indicates that the DC between segments did not differ.

Table S3.3. Cumulative retention times (CRT, min) of digesta recovered from the small intestine of pigs fed barley, maize or high amylose maize based diets which included starch as isolated powder, ground cereal or extruded cereal[1].

	Experimental diets								
	Barley			Maize			High amylose maize		
	Isolated	Ground	Extruded	Isolated	Ground	Extruded	Isolated	Ground	Extruded
Max obs[2]	10	10	9	10	10	9	7	7	10
SI1	3	4	3	4	4	2	3	3	3
SI2	15	19	16	19	20	12	20	19	17
SI3	50	66	50	61	55	49	56	56	57
SI4	91	117	90	106	90	96	95	98	101

[1] 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).
2 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.

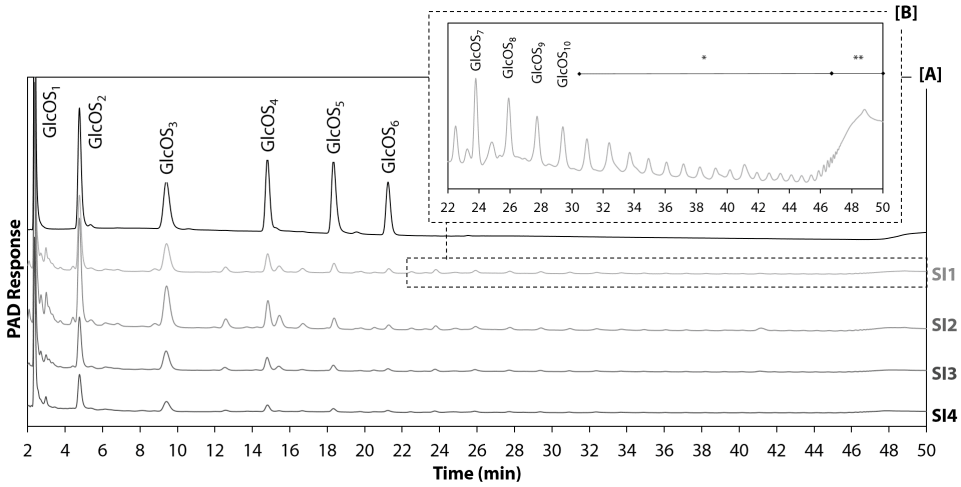


Figure S3.1. Typical soluble oligosaccharide profile in small intestinal digesta of pigs (pooled digesta of 10 pigs) fed ground maize. Panel [A]: The top line represents an HPAEC elution pattern of α (1-4) gluco-oligosaccharides (GlcOSn) with a known concentration, which vary in the number of glucose units (n), used to quantify oligosaccharide concentrations. Other lines show the elution pattern of soluble supernatant of pooled digesta recovered from four small intestinal segments (SI1-SI4). Panel [B]: Enlarged elution profile (22 to 50 min) from which longer oligosaccharides could be identified. * Indicates individual gluco-oligosaccharides, ** indicates soluble starch derived fragments which could not be separated and are therefore quantified as group.

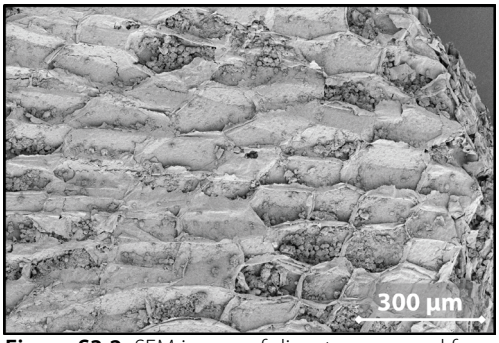
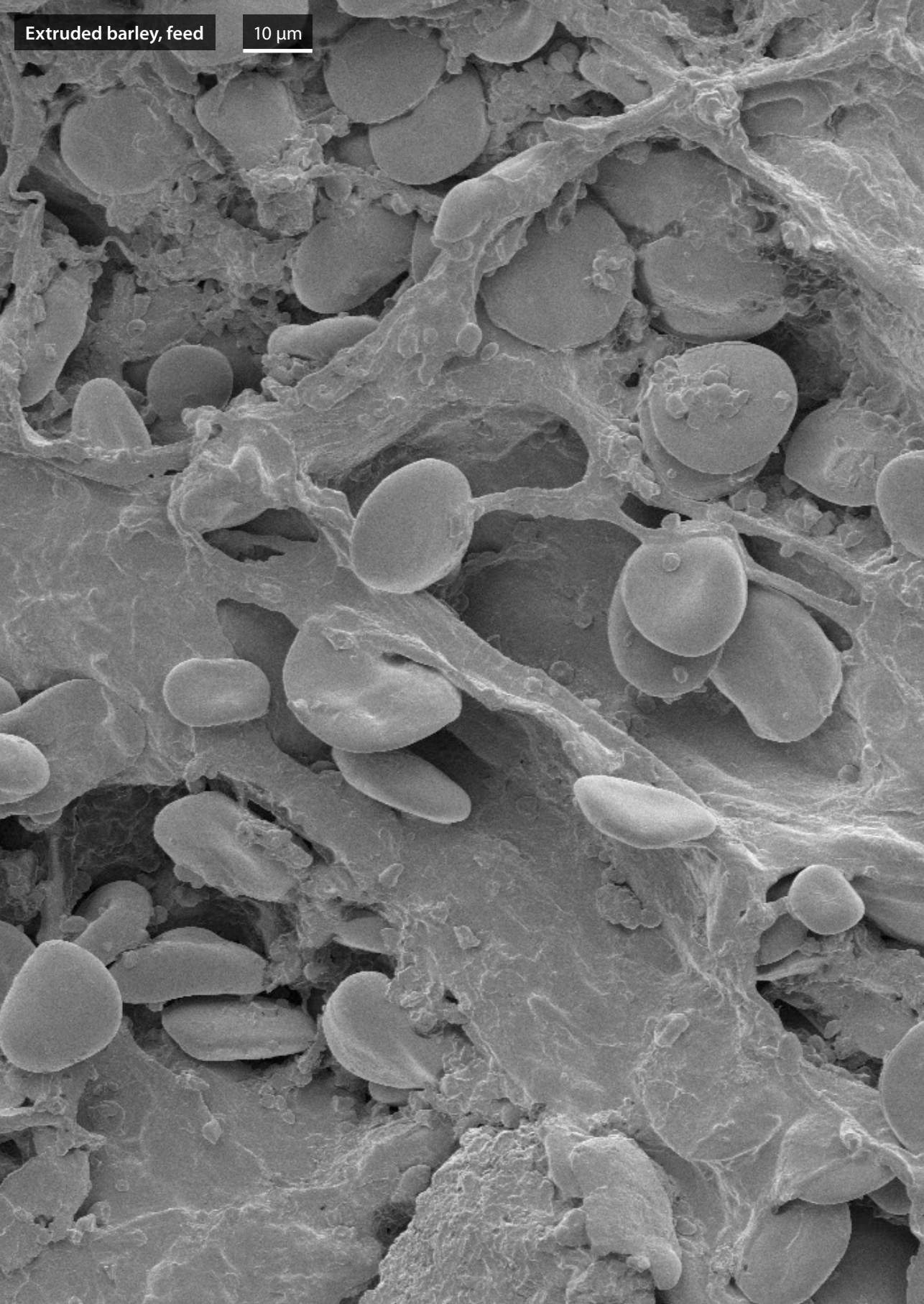


Figure S3.2. SEM image of digesta recovered from SI4 of a pig fed ground maize, 250x magnified.


Extruded barley, feed
10 µm

Chapter 4

Whole digesta properties as influenced by feed processing explain variation in gastrointestinal transit times in pigs

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Abstract

Physicochemical properties of diets are believed to play a major role in the regulation of digesta transport in the gastro-intestinal tract. Starch, being the dominant nutrient in pig diets, strongly influences these properties. We studied transport of digesta solids and liquids trough the upper gastro-intestinal tract of 90 pigs in a 3x3 factorial arrangement. Dietary treatments varied in starch source (barley, maize, high amylose maize) and form (isolated starch, ground cereal, extruded cereal). Mean retention times (MRT) of digesta solids ranged from 129-225 min for the stomach and from 86-124 min for the small intestine (SI). The MRT of solids consistently exceeded that of liquids in the stomach, but not in the SI. Solid digesta of pigs fed extruded cereals remained 29-75 min shorter in the stomach compared with pigs fed ground cereals ($P<0.001$). Shear stress of whole digesta positively correlated with solid digesta MRT in the stomach ($r=0.33$, $P<0.001$), but not in the SI. The saturation ratio (SR), the actual amount of water in stomach digesta as a fraction of the theoretical maximum hold in the digesta matrix, explained more variation in digesta MRT than shear stress. The predictability of SR, however, was hampered by the accumulation of large particles in the stomach. In addition, the water holding capacity of gelatinised starch lead to a decreased SR of diets, but not of stomach digesta, which was caused by gastric hydrolysis of starch. Both of these phenomena hinder the predictability of gastric retention times based on feed properties.

Introduction

Pig performance is affected by the rate of nutrient appearance in the portal vein. For example, pigs fed diets rich in rapidly digestible starch have shorter inter-meal intervals and meal durations[1] and greater activity-related energy expenditure[2], compared with pigs fed slowly digestible or resistant starch. Additionally, feeding pigs free lysine, which is rapidly absorbable, leads to a greater oxidation of essential amino acids compared with feeding protein-bound lysine[3]. The rate of nutrient absorption is affected mostly by the rate of hydrolysis in combination with digesta transport, especially through the stomach and proximal small intestine (SI)[4]. The rate at which digesta are transported through those organs is, in turn, affected by several mechanisms and meal properties, such as meal size[5], caloric content[6], and nutrient-activated feedback mechanisms[7,8]. Moreover, digesta transport depends on the composition and properties of digesta. Typically, digesta are complex particulate suspensions, which change continuously upon transfer through the gastro-intestinal tract (GIT)[9]. Whole digesta consists of a soluble fraction and insoluble particle fraction that travel at a different speeds through the GIT[10,11]. Consequently, nutrient absorption kinetics depend on the solubility of nutrients. Transit behaviour of whole digesta can be characterised by measuring the rheological properties of digesta, which depend on several basic chemical and physical properties of both the solid and liquid fractions[9,12-14]. For example, rheological properties of whole digesta depend on the DM content, the concentrations of soluble and insoluble polymers, liquid fraction viscosity, and several properties related to the particular matter, such as its size distribution, WHC, and deformability[9,12-15]. These properties can affect the MRT of various digesta fractions. For example, large particles (>1-2 mm) remain in the human[16,17] and canine[18] stomach until they are broken down to smaller particles, thereby increasing the gastric retention time of digesta solids. In addition, a high viscous liquid fraction of digesta reduces solid digesta passage rates in humans[19] and pigs[20] in the upper GIT. Data on the relation between whole digesta rheology and its underlying properties, however, is scarce, and relations between whole digesta properties and transport are poorly understood[10,15,21].

Starch, in many pig diets provided in the form of cereals, is quantitatively the most important macronutrient and typically represents 40-50% of the diet[22]. The form in which starch is presented to the pig, is therefore one of the main determinants of rheological properties of diets. For example, feed processing such as pelleting or extrusion, typically results in fractions of gelatinised starch[23,24], which increases the liquid fraction viscosity[25]. In addition, rheological properties of non-hydrothermal treated diets are affected by milling conditions, as the particle size distribution and shape affects the maximum packing density of solids in the composite suspension, which in turn affects digesta viscosity[9]. In the present study we assessed digesta passage behaviour throughout the upper GIT of pigs fed one of nine diets, varying in starch form and source. In addition, we studied relationships between whole digesta rheology and digesta MRT. The correlation between rheology and MRT was further explored by examination of underlying physical digesta properties. Lastly, we investigated the prediction of stomach digesta properties based on feed properties.

We hypothesized that whole digesta rheological properties would explain a major fraction of variation in digesta MRT. Hydrothermal processing of cereals by extrusion will lead to starch gelatinization and a reduction in average particle size. The first is expected to increase digesta MRT in pigs, whereas the latter is expected to decrease MRT. The net effect therefore remains unknown.

Materials and methods

Experimental design, animals, and diets

The experiment described in the present manuscript was part of a larger study on starch digestion kinetics, which is described in detail elsewhere[26]. The experiment was approved by the Dutch Central Committee of Animal Experiments under the authorization number AVD260002016550. Briefly, 90 crossbred gilts (Topigs 20 × Pietrain sire), weighing 23.1 ± 2.1 kg, were assigned to one of nine dietary treatments in a 3 x 3 factorial arrangement, in four successive batches. Factors were starch source (barley, maize, high amylose (HA) maize) and form (isolated starch, ground cereal, extruded cereal). The resulting dietary treatments were: 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 (EA) form. In total, 96 pigs were used: 10 pigs were assigned per dietary treatment, whereas the remaining animals served as reserve animals and were used to replace excluded animals. Fourteen pigs were excluded due to a low feed intake: pigs that were excluded in one of the first three batches were replaced in the sequential batch. Replacement was done in such a way 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 one. The experiment consisted of an adaptation period of at least 2 days, 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 but fed individually at 2.0 × the energy requirements for maintenance (750 kJ NE per kg BW^0.60)[27]. All the diets were mixed with water just before feeding. In the first batch, all diets were mixed with water to a feed:water ratio 1:2. After the first batch, the feed:water ratio of ground diets was altered to 1:1.5 to facilitate ingestion. 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 1/12th 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 euthanised. Pigs were euthanised and dissected in an order balanced for dietary treatment and time after onset of the frequent feeding procedure. Upon the start of the frequent feeding procedure of the first pig, extra meals (1/12th 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. Diets were formulated to meet or exceed the nutrient requirements of growing pigs[27] and designed to contain ~400 g starch per kg dry feed. All diets were formulated to be identical in crude protein, fat and total dietary fibre content, using soybean meal, -hulls, -protein isolate, -oil, and sugar beet pulp. Details on ingredients, production conditions, and the analysed composition are described elsewhere[26]. Chrome (Cr) and Cobalt (Co) were included as markers in the feed at a level of 170 mg/kg (w/w, as fed basis), in the form of chromium oxide (Cr2O3) and Co-EDTA, respectively.

Digesta collection

Prior to dissection, pigs were sedated and exsanguinated as described in detail elsewhere[26]. Immediately after exsanguination, clamps were placed between gastro-intestinal sections to prevent the movement of digesta, and the GIT was carefully removed. The stomach content was homogenised by manual mixing and after recording the total weight and the pH, samples were collected. One representative sample was immediately frozen and kept at –20°C until freeze drying, whereas another sample was kept at 4°C pending rheology and particle size analyses. The small intestine was carefully spread on a table and divided with clamps in four segments. The last 1.5 m from the small intestine (SI4) was considered to represent the terminal 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 and kept at –20°C until freeze drying. In addition, samples from SI2 and SI4 were taken and stored at 4°C pending rheology and particle size analyses.

Chemical, physical, and rheological analyses

Prior to chemical analyses, feed and freeze dried digesta samples were ground to pass a 1 mm sieve using a centrifugal mill at 12 000 rpm (ZM200; Retsch, Haan, Germany). All analyses were performed in duplicate, 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 according to NEN-ISO 6496[28]. Total starch content of all diet and digesta samples was determined in triplicate according to AOAC Method 996.11 with the total starch assay kit from Megazyme (Wicklow, Ireland).

Viscosity of digesta was measured using stress-controlled rheometers (MCR 301/MCR 502, Anton Paar GmbH, Graz, Austria) in samples (<48 h after digesta collection, stored at 4°C), without separation of the liquid and solid fraction, at 39°C, as described previously[14] with slight adjustments. Briefly, feed samples were analysed after soaking the feed for 1 h in the feed:water ratio as fed from batch 2 onwards (1:2 for diets with isolated starch and extruded cereals, 1:1.5 for diets with ground cereals). A parallel plate profiled geometry (PP25/P2) of 25 mm diameter with a ribbed surface was used to avoid slip and a plastic lid was used to avoid

evaporation. For small intestinal digesta samples, harvested from the second and last part of the small intestine, the apparent viscosity curve was measured using a frequency sweep (100 to 1 Hz log). Gel strengths of feed and stomach digesta were too high to measure the viscosity curve. To ensure permanent contact and confinement pressure, those samples were subjected to an oscillatory frequency sweep (from 275 to 1 Hz) at normal force controlled gap distance (0.5 N) and a constant strain (10%). Settings were optimised based on the sample that had visually the highest gel strength, which was stomach digesta originating from pigs fed diets with isolated starches. For stomach digesta recovered from pigs fed EB or EM, the gel strength was not sufficient to remain a constant normal force controlled gap distance. Therefore, samples were subjected to the oscillatory frequency sweep at a fixed gap distance (2 mm). With the oscillatory measurements we identified digesta shear stress, storage modulus (G′), and loss modules (G″) at a frequency of 1 Hz, as previous research suggested that the forces naturally applied by the GIT are close to this frequency[29-31].

Particle size of digesta was analysed at 20°C in samples that were stored at 4°C or -21°C. Feed samples were analysed after soaking for 1 h in the feed:water ratio as fed from batch 2 onwards. Particle size was measured by laser diffraction (Mastersizer 3000; Malvern, Worcestershire, UK) using demineralized water as dispersant. The reference material was wood flour (refraction index 1.53, absorption index 0.1, as supplied by the manufacturer) and each sample was analysed at least in triplicate. Measurements were performed in the range 0.01 to 3500 µm. For further analyses, the volume percentage of particles was summarised in 3 classes: small particles, between 3.5-35 µm, medium particles, 35-350 µm, and large particles, 350-3500 µm.

Water holding capacity (WHC) of diets and freeze dried digesta was determined in ground material using the Baumann's apparatus[32]. A total of 105±6 mg of ground and freeze dried sample was placed on a filter disc of 40 mm diameter and 10-16 µm pore size (Duran group, Mainz, Germany). The volume of water absorbed to hydrate the sample until saturation was recorded and corrected for the amount of water that evaporated in this time, which was determined using a filter disc without sample.

Chromium (Cr) and cobalt (Co) concentrations were measured in singlicate in feed and digesta by inductively coupled plasma optical emission spectroscopy. Cr and Co were measured at 357.9 and 228.0 nm respectively according to Van Bussel et al.[33], after sample preparation according to Williams et al[34].

Molecular weight distributions of the soluble fractions of feed and digesta were analysed with High Performance Size Exclusion Chromatography (HPSEC). Digesta from all pigs within a dietary treatment were pooled on weight base by mixing the same quantity of freeze dried digesta from each pig together. Freeze dried and ground diets and pooled digesta were boiled in water for 5 minutes (50 mg/ml) and subsequently centrifuged. Supernatant was analysed using an Ultimate 3000 HPSEC system (Thermo Fisher Scientific, Waltham, MA, USA). A set of four TSK-Gel columns (Tosoh Bioscience, Tokyo, Japan) was used in series: one guard column (6 mm ID × 40 mm) and the columns super AW4000, 3000 and 2500 (6 mm × 150 mm). The column temperature was set to 55°C. 10 µL sample was eluted with filtered 0.2 M $NaNO_3$ at a

flow rate of 0.6 ml/min and the elution was monitored by refractive index detection (Shodex RI 101; Showa Denko K.K., Kawasaki, Japan).

Statistics and calculations

The MRT of solid and liquid fractions of digesta was calculated based on quantities of Cr and Co recovered in GIT segments, assuming that hourly feeding induced steady state conditions, according to the following equation:

Equation 4.1.

$$MRT\ (n) = \frac{300 * [marker] * W}{I}$$

Where MRT is the mean retention time in minutes in compartment *n* of the GIT; [marker] is the marker (Cr or Co) concentration in the digesta (mg/g DM); W is the weight of the dry intestine content (g DM) and I is the marker intake over 300 min prior to dissection (mg). ΔMRT was calculated as digesta MRT of solids minus the digesta MRT of liquids at each GIT compartment.

The power law model was used to model the apparent viscosity of small intestinal digesta, per pig per segment, measured over a range of shear rates[36]:

Equation 4.2.

$$Apparent\ viscosity = K * shear\ rate^{(n-1)}$$

Where K is the consistency coefficient (pa*s^n), which reflects the shear stress at a shear rate of 1/s, and n is the flow behaviour index, which is dimensionless and reflects the closeness to Newtonian flow. K and n were estimated by nonlinear regression procedures (PROC NLIN, SAS, version 9.4, SAS Institute, Cary, USA).

To characterise the rheological properties of diets and stomach digesta, tanδ was calculated according to the following equation[37]:

Equation 4.3.

$$tan\delta = \frac{loss\ modulus}{storage\ modulus}$$

Where loss and storage moduli were measured at 1 Hz.

From the DM content and WHC of diets and digesta, we calculated the saturation ratio (SR). The SR is the digesta water content, as fraction of the theoretical maximum of water that can be held by the dry matter according to its WHC. The SR was calculated according to the following equation:

Equation 4.4.

$$Saturation\ ratio = \frac{Water\ content}{Max\ water\ held}$$

Where the water content is the percentage of water in the dietary or digesta suspension.

Max water held is the amount of water that can maximally be held in the dietary or digesta suspension, calculated as the DM content times WHC. For diets, the DM represents the DM content of diets after they were mixed with water, in the ratios applied prior to feeding. An SR < 1 indicates that less water is present in the stomach than the amount of water that can potentially be held by the amount of DM. An SR > 1 indicates that more water is present in the stomach than can be hold by the digesta matrix, based on its WHC properties.
Effects of dietary treatments on MRT were tested using a general linear mixed model (PROC MIXED, SAS), with starch form, starch source, and their interaction, as fixed effects and batch as random effect. Least square means were compared after Tukey's adjustment for multiple comparisons. Correlation coefficients between whole digesta rheology parameters and MRT, and whole digesta rheology and physical properties, were estimated using Pearson's correlation procedure (PROC CORR, SAS). Data are presented as least squares (LS) means and pooled standard deviation of the mean (S) unless stated otherwise. A retrospective power analysis was performed to validate the sample size of this study. Considering digesta MRT as the most important parameter, the power was evaluated using the variation in digesta MRT 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[38]. For the stomach and small intestine, a power greater than 0.69 was reached on the main effect of starch form and a power greater than 0.52 was reached on the main effect of starch source. For the form x source interaction, a power of 0.29 was reached for the stomach and a power of 0.72 was reached for the small intestine. Significance was assumed at $P \leq 0.05$, while a tendency was considered when $0.05 < P \leq 0.1$.

Results

Mean retention times of solid and liquid digesta

The MRT of solid stomach digesta was 29 to 75 min shorter for pigs fed extruded cereals, compared with pigs fed ground cereals ($P<0.01$, **Table 4.1**). The inclusion of barley tended to reduce the MRT of both solids (35-39 min) and liquids (28-29 min) in the stomach, when compared with maize and HA maize ($P<0.1$). The effects of dietary treatment on the separation of digesta fractions in the stomach were studied by subtracting the liquid MRT from the solid MRT (ΔMRT, **Table 4.2**). Extrusion reduced the ΔMRT in the stomach of pigs fed barley and maize on average by 59 min, compared with diets containing ground cereals, which was not observed for pigs fed HA maize (form x source, $P<0.001$).
In the small intestine, the MRT of solid digesta averaged 7 min in SI1, 22 min in SI2, 51 min in SI3, and 28 min in SI4 (**Table 4.1**). The cumulative MRT of solid digesta in the SI of pigs fed GB (124 min) was longer than observed for IB (86 min, form x source, $P<0.05$). This effect of form was not present within pigs fed either maize or HA maize. The MRT of liquid digesta exceeded that of solid digesta in the SI for all pigs, except those fed EB ($P<0.05$, **Table 4.2**). The ΔMRT in the SI tended to be longer for pigs fed diets with ground cereals, compared with pigs fed extruded cereals ($P<0.1$).

Table 4.1. Mean retention times (MRT, min) of solid and liquid fractions of digesta recovered from the stomach and 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]

	Experimental diets									S	P – value[4]		
	Barley			Maize			High amylose maize						
	Isolated	Ground	Extruded	Isolated	Ground	Extruded	Isolated	Ground	Extruded		Form	Source	Form*Source
Max obs[5]	10	10	9	10	10	9	7	7	10				
Digesta solids													
Stomach	161^{kl}	197^{k}	129^{l}	225^{kl}	221^{k}	146^{l}	190^{kl}	221^{k}	192^{l}	66	**0.008**	*0.062*	0.437
SI 1	7	8	5	8	8	5	7	5	7	4	0.394	0.691	0.594
SI 2	15	25	22	21	23	17	27	27	22	11	0.201	0.196	0.496
SI 3	46	63	43	53	45	56	43	48	59	14	0.355	0.969	0.004
SI4	21	29	24	18	28	34	33	39	30	14	0.129	*0.053*	0.302
Total SI	86^{b}	124^{a}	94^{ab}	100^{ab}	102^{ab}	111^{ab}	109^{ab}	120^{ab}	116^{ab}	22	**0.024**	*0.073*	0.039
Digesta liquids													
Stomach	132	127	130	187	159	131	171	164	137	55	0.132	*0.089*	0.652
SI 1	7	7	5	9	8	4	7	5	6	5	*0.071*	0.735	0.740
SI 2	18	24	21	22	24	16	29	29	25	11	0.278	**0.049**	0.793
SI 3	61	78	52	67	56	70	53	67	70	20	0.532	0.977	0.014
SI4	28^{y}	35^{y}	24^{y}	23^{xy}	33^{xy}	36^{xy}	38^{x}	51^{x}	37^{x}	18	0.148	**0.025**	0.513
Total SI	111^{y}	143^{y}	101^{y}	121^{xy}	118^{xy}	125^{xy}	127^{x}	152^{x}	137^{x}	30	*0.060*	**0.038**	0.108

[1] Presented values are estimated LSMeans and pooled standard deviation (S).

[2] MRT are estimated based on quantities of Cr (solids) and Co (liquids) recovered from digesta.

[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). Total SI represents the total small intestine.

[4] p-values for fixed effects of starch form (isolated, ground, vs extruded), source (barley, maize, vs. high amylose maize), and the interaction between form and source, analysed per segment. When an interaction between form and source was found (P<0.05), superscripts ^{a,b} indicate significant differences between treatment combinations (P<0.05). In the absence of source*form interactions, superscripts ^{k,l} are used to indicate significant differences between starch forms (P<0.05) and superscripts ^{x,y} indicate significant differences between starch sources (P<0.05).

[5] The maximum number of replicate observations equals the number of replicate animals per treatment. In some segments, not enough digesta were 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.

Table 4.2. Difference between mean retention times of solid and liquid fractions of digesta (ΔMRT, min) recovered from the stomach and 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]

	Experimental diets									S	P – value[4]		
	Barley			Maize			High amylose maize						
	Isolated	Ground	Extruded	Isolated	Ground	Extruded	Isolated	Ground	Extruded		Form	Source	Form*source
Max obs[5]	10	10	9	10	10	9	7	7	10				
Stomach	29^{abcd}*	69^{a}*	-1^{d}	38^{abcd}*	62^{ab}*	15^{cd}	19^{bcd}	57^{abc}*	54^{abc}*	29	**<0.0001**	0.401	**0.003**
SI 1	-1^{l}	1^{k}	1^{k}	-1^{l}	0^{k}	1^{k}	-1^{l}	0^{k}	1^{k}	2	**0.004**	0.808	0.776
SI 2	-3	2	0	-1	-1	2	-2	-2	-3	6	0.544	0.375	0.476
SI 3	-15*	-15*	-9*	-15*	-10*	-14*	-9*	-18*	-11*	9	0.444	0.994	0.186
SI4	-6*	-6*	1	-4	-5	-2	-6	-11*	-7*	8	0.115	0.117	0.559
Total SI	-25*	-18*	-7	-21*	-16*	-14*	-18*	-32*	-21*	14	**0.065**	0.168	0.126

[1] Presented values are estimated LSMeans and pooled standard deviation (S).

[2] ΔMRT is calculated as MRT of the solid digesta fraction minus MRT of the liquid digesta fraction, which are estimated based on quantities of Cr and Co, respectively.

[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] p-values for fixed effects of starch form (isolated, ground vs extruded), source (barley, maize vs. high amylose maize), and the interaction between form and source, analysed per segment. When an interaction between form and source was found, superscripts ^{a,b} indicate significant differences between treatment combinations (P<0.05). In the absence of source*form interactions, superscripts ^{kl,} are used to indicate significant differences between starch forms (P<0.05) and superscripts ^{xy} indicate significant differences between starch sources (P<0.05). * indicates whether ΔMRT differed from zero (P<0.05).

[5] The maximum number of replicate observations equals the number of replicate 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.

Rheological characterization of feed and digesta

All experimental diets had a storage modulus that exceeded the loss modulus and consequently a tanδ between 0 and 1 (**Table 4.3**). Extrusion increased the dietary shear stress of barley diets by a factor 1.9 and maize by a factor 1.6, whereas this was only a factor 1.3 for HA maize.

Regardless of the diet fed, tanδ of stomach digesta was between 0 and 1. The shear stress of all isolated and ground diets increased upon ingestion, whereas it decreased upon ingestion for extruded diets, except for HA maize. Within pigs fed ground cereals, stomach digesta of pigs fed barley had a higher shear stress than those fed maize or HA maize (form x source, $P<0.001$). The shear stress of stomach digesta was greater for pigs fed isolated and ground diets, than for pigs fed extruded diets, particularly for pigs fed barley and maize ($P<0.001$).

For all dietary treatments, the SI digesta viscosity at 1 s^{-1}, equalling K, increased from SI2 to SI4. For SI2, pigs fed IM had a higher digesta viscosity than pigs fed EM, which was not observed for pigs fed barley and HA maize (form x source, $P<0.05$). Additionally, digesta viscosity of SI2 of pigs fed GA maize exceeded that of EA, whereas this difference was absent for maize and barley fed pigs (form x source, $P<0.05$). In SI4, digesta of pigs fed isolated diets had a higher viscosity (on average 227 pa*s, $P<0.05$) compared with pigs fed ground (155 pa*s) and extruded diets (140 pa*s). Additionally, pigs fed GB tended to have a lower digesta viscosity in SI4 than pigs fed IB (form x source, $P=0.08$).

Correlations between digesta MRT and rheology of diets and whole digesta

Dietary shear stress was negatively correlated with solid digesta MRT in the stomach ($r=-0.71$, $P<0.05$, **Table 4.4**). In the stomach, digesta shear stress was positively correlated with solid digesta MRT ($r=0.33$, $P<0.001$), but not with liquid digesta MRT. In contrast, digesta viscosity in both SI2 and SI4 explained almost no variation in solid nor liquid digesta MRT ($r<0.10$, $P>0.1$). To further unravel the correlation between digesta rheology and MRT, we examined underlying physical and chemical properties of diets and stomach digesta, but not of small intestinal digesta.

Table 4.4. Pearson correlation coefficients for digesta MRT and rheological properties, of diets, stomach, and small intestinal digesta[1].

	MRT_{solid} stomach	MRT_{liquid} stomach	MRT_{solid} SI2	MRT_{liquid} SI2	MRT_{solid} SI4	MRT_{liquid} SI4
Shear stress feed	-0.71*	-0.47	-0.22	-0.50	-0.05	-0.37
Shear stress stomach digesta	0.33***	0.19				
K SI2 digesta			0.02	0.07		
K SI4 digesta					0.02	0.09

[1]* indicates P<0.05, ** indicates P<0.01, *** indicates P<0.001

Table 4.3. Rheological properties of feed and digesta recovered from the stomach and 2 parts of the small intestine of pigs fed diets differing in starch source (barley, maize or high amylose maize) and form (as isolated powder, ground cereal or extruded cereal)[1,2,3,4].

		Experimental diets									S	P – value[5]		
		Barley			Maize			High amylose maize						
		I	G	E	I	G	E	I	G	E		form	source	F x S
Diet	**Shear stress (pa)**	147	238	445	202	213	344	166	152	196				
	Storage modulus (pa)	1337	2067	4134	1853	1886	3045	1544	1371	1768				
	Loss modulus (pa)	604	1180	1634	809	995	1601	603	664	838				
	tanδ	0.45	0.57	0.40	0.44	0.53	0.53	0.39	0.48	0.47				
Max obs[6]		10	10	9	10	10	9	7	7	10				
Stomach	**Shear stress (pa)**	590^{ab}	620^{a}	20^{e}	550^{abc}	460^{c}	0^{e}	570^{abc}	480^{bc}	200^{d}	84	**<0.0001**	**0.020**	**<0.0001**
	Storage modulus (pa)	5194	5177	108	4933	4095	20	4993	4254	1840				
	Loss modulus (pa)	2726	3242	49	2460	2016	9	2417	2000	751				
	tanδ	0.53^{b}	0.62^{a}	0.48^{bc}	0.49^{bc}	0.49^{bc}	0.50^{bc}	0.48^{bc}	0.47^{c}	0.41^{d}	0.036	**<0.0001**	**<0.0001**	**<0.0001**
SI2	**K (Pa*s)**	98^{abc}	85^{bc}	62^{bc}	118^{ab}	64^{bc}	34^{c}	132^{ab}	167^{a}	62^{bc}	39	**<0.0001**	**0.001**	**0.012**
	n	0.009^{l}	0.004^{l}	0.020^{k}	0.005^{l}	0.045^{l}	0.118^{k}	0.007^{l}	0.007^{l}	0.078^{k}	0.062	**0.004**	*0.066*	0.308
SI4	**K (Pa*s)**	251^{k}	110^{l}	142^{l}	210^{k}	137^{l}	158^{l}	220^{k}	216^{l}	120^{l}	75	**0.001**	0.708	*0.083*
	n	-0.001	0.002	0.001	0.000	0.000	0.000	0.011	-0.001	0.012	0.014	0.668	0.234	0.563

[1] Presented values for diet samples are averages of four measurements.

[2] Presented values for digesta samples are estimated LSMeans and pooled standard deviation (S), except for the storage and loss moduli, which are raw means.

[3] Shear stress, tanδ and K are measured at 1 Hz

[4] Abbreviations: I, isolated powder. G, ground cereal. E, extruded cereal. F, form. S, source.

[5] Model established p-values for fixed effects of starch form (isolated, ground vs extruded), source (barley, maize vs. high amylose maize), and the interaction between form and source, analysed per segment. When an interaction between form and source was found, superscripts ^{a,b,c} indicate significant differences between treatments (P<0.05). In the absence of source*form interactions, superscripts ^{k,l,m} are used to indicate significant differences between starch forms (P<0.05).

[6] The maximum number of replicate observations (max obs) equals the number of replicate animals per treatment. In some segments, not enough digesta was present to allow analysis, causing one missing observation in the stomach of pigs fed EM and SI2 of pigs fed GM, IA, GA, and EA, two missing observations in SI2 of pigs fed EB and SI4 of pigs fed GM, EM, and EA, three missing observations in SI2 of pigs fed IB and IM and SI4 of pigs fed GB and IM, and four missing observations in SI2 of pigs fed EM and SI4 of pigs fed IB and EB.

Table 4.5. Physical properties of feed and digesta recovered from the stomach of pigs fed diets differing in starch source (barley, maize or high amylose maize) and form (as isolated powder, ground cereal or extruded cereal)[1,2,3].

		Experimental diets									S	P – value[4]		
		Barley			Maize			High amylose maize						
		I	G	E	I	G	E	I	G	E		form	source	F x S
Feed														
	3.5 - 35 µm (%)	17	6	5	14	6	10	19	6	4				
PSD	35 - 350 µm (%)	39	75	65	43	73	63	44	76	73				
	350 - 3500 µm (%)	43	19	30	42	21	27	35	19	24				
WHC (mL/g)		2.1	2.1	4.2	2.1	1.9	3.4	2.4	1.9	2.5				
DM content (%)		91	91	96	90	91	96	90	91	96				
SR		1.11	0.85	0.51	1.11	0.94	0.63	0.97	0.94	0.85				
pH		6.6	7.0	7.0	6.7	6.9	6.9	6.6	6.8	6.9				
Stomach digesta														
Max obs[5]		10	10	9	10	10	9	7	7	10				
	3.5 - 35 µm (%)	13	20	23	13	12	19	13	12	18				
PSD	35 - 350 µm (%)	16	25	38	16	29	33	15	2	25				
	350 - 3500 µm (%)	$70^{k\|y}$	$52^{l\|y}$	$37^{m\|y}$	$70^{k\|xy}$	$58^{l\|xy}$	$47^{m\|xy}$	$70^{k\|x}$	$65^{l\|x}$	$55^{m\|x}$	4.5	**<0.0001**	**<0.001**	*0.074*
WHC (mL/g DM)		3.3^{k}	2.3^{l}	2.2^{l}	3.2^{k}	2.2^{l}	2.3^{l}	3.5^{k}	2.0^{l}	2.1^{l}	0.3	**<0.0001**	0.812	0.248
DM content (%)		23^{cd}	32^{a}	22^{cd}	25^{c}	35^{a}	21^{d}	24^{cd}	33^{a}	29^{b}	2.3	**<0.0001**	**<0.001**	**<0.0001**
SR		1.02^{bc}	0.93^{c}	1.66^{a}	1.00^{bc}	0.90^{c}	1.73^{a}	0.92^{c}	0.98^{bc}	1.23^{b}	0.19	**<0.0001**	0.003	**<0.001**
pH		4.3^{ab}	4.1^{ab}	4.6^{a}	4.0^{ab}	4.3^{ab}	3.9^{ab}	3.6^{b}	4.7^{a}	4.2^{ab}	0.5	**0.047**	0.178	**0.008**

[1] Presented values for diet samples are averages of duplicate measurements.

[2] Presented values for digesta samples are estimated LSMeans and pooled standard deviation (S).

[3] Abbreviations: isolated powder (I), ground cereals (G), extruded cereals (E), form (F), source (S), particle size distribution (PSD), water holding capacity (WHC), dry matter content (DM), and saturation ratio (SR).

[4] Model established p-values for fixed effects of starch form (isolated, ground vs extruded), source (barley, maize vs. high amylose maize), and the interaction between form and source, analysed per segment. When an interaction between form and source was found, superscripts [a,b,c] indicate significant differences between treatments ($P<0.05$). In the absence of source*form interactions, superscripts [k,l,m] are used to indicate significant differences between starch forms ($P<0.05$) and superscripts [x,y,z] indicate significant differences between starch sources ($P<0.05$).

[5] The maximum number of replicate observations equals the number of replicate animals per treatment. For WHC, DM and pH, the actual number of observations equals the maximum number of observations. For some animals, not enough digesta was collected and stored fresh to allow particle size analysis, causing one missing observation in pigs fed EB, IM, GM, IA, GA, and EA, two missing observations in pigs fed GB, three missing observations in pigs fed IB, and four missing observations in pigs fed EM.

Physical and chemical properties of feed and stomach digesta

The particle size distributions of feed and digesta samples were characterized by the presence of three distinct peaks for all samples. As a representative example, particle size distributions of feed and stomach digesta from IB, GB and EB treatments are represented in **Figure 4.1**. Diets with ground and extruded cereals consisted mainly out of medium sized particles (71 vol% on average), whereas diets with isolated starch had a rather equal distribution of medium (42 vol% on average) and large particles (40 vol% on average, **Table 4.5**). Stomach digesta consisted mainly of particles larger than 350 µm. As expected, the particle size fractions within diets and stomach digesta were highly correlated (**Table 4.6**). Dietary treatment effects on the particle size distribution of stomach digesta were therefore analysed for the large particle size fraction only. Stomach digesta of pigs fed ground diets contained more large particles (58 vol% on average) compared with that of pigs fed extruded diets (46 vol% on average), but less than pigs fed isolated diets (70 vol% on average, $P<0.001$). Stomach digesta of pigs fed HA maize contained more large particles (63 vol% on average) than pigs fed barley (53 vol% on average, $P<0.001$).

Water holding capacity (WHC, **Table 4.5**) of dry diets was comparable for diets containing isolated starch (2.2 mL/g) and ground cereals (1.9 mL/g). Extrusion increased the WHC with 2.1 mL/g for barley, 1.5 mL/g for maize, and 0.6 mL/g for HA maize, compared with ground cereals. Stomach digesta of pigs fed diets with isolated starch had a higher WHC (3.4 mL/g) than those of pigs fed ground and extruded diet (both 2.2 mL/g, $P<0.001$).

Differences in stomach DM content were dominated by a higher digesta DM content for pigs fed ground diets compared with those fed isolated and extruded diets, particularly for barley and maize diets (form x source, $P<0.001$, **Table 4.5**). The SR of diets was slightly above 1 for IB and IM, whereas it was below 1 for all other diets. The SR of stomach digesta obtained from pigs fed extruded cereals was higher than for pigs fed diets containing isolated starches or ground cereals, except for diets from high amylose maize origin (form x source, $P<0.001$, **Table 4.5**). For diets from high amylose maize origin, the SR of stomach digesta from pigs fed extruded cereals was higher than for pigs fed diets with isolated starch, but not for pigs fed ground cereals (form x source, $P<0.001$, **Table 4.5**).

Upon ingestion, the pH decreased on

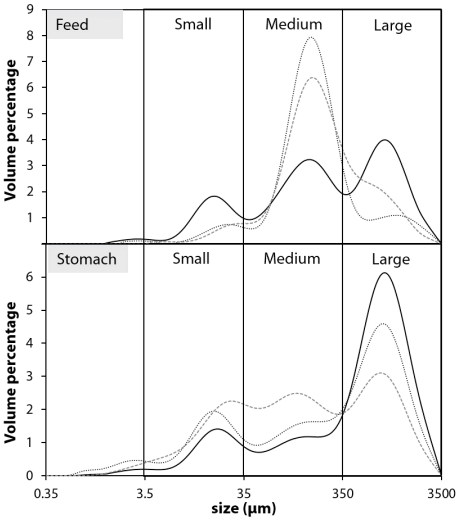

Figure 4.1. Typical particle size distribution of barley based diets, visualised for feed (top frame) and stomach digesta (bottom frame), which included isolated starch (solid line), ground cereals (dotted line) or extruded cereals (dashed line).

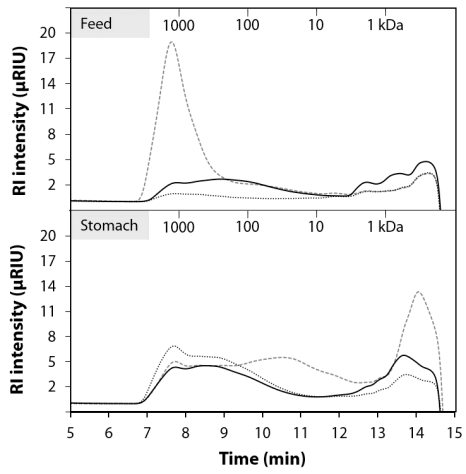

Figure 4.2. Soluble polysaccharide profile of maize based diets, which included isolated starch (solid line), ground cereals (dotted line) or extruded cereals (dashed line), visualised for feed (top frame) and stomach digesta (bottom frame), as measured with high performance size exclusion chromatography. The second x-axis indicates the molecular weight calibration curve for pullulan.

average with 2.6 unit points to 4.2 unit points (**Table 4.5**). Stomach pH was affected by an interaction (P<0.01) between form and source of starches used. The pH of stomach digesta for pigs fed IA was lower than that of pigs fed GA (form x source, P<0.05), whereas this difference was not observed for pigs fed barley or maize.

Soluble polymers in a water extract of feed and stomach digesta were analysed with HPSEC. A representative HPSEC profile is presented for maize starch in isolated, ground and extruded form, in **Figure 4.2**. Diets with extruded cereals had the highest concentration of large soluble polymers (MW ~ 1000 kDa). Upon ingestion, the concentration of large polymers decreased, whereas an increase in small polymers (MW ~ 1 kDa) was identified, especially for pigs fed extruded cereals. High Performance Anion Exchange Chromatography (HPAEC) revealed the presence of maltodextrines DP 2-6 as typical breakdown products of starch (data not shown), accounting for 18% of total starch in stomach digesta of pigs fed diets containing extruded cereals and for <5% for pigs fed diets with ground cereals and isolated starch.

Correlations between rheological and physical properties of diets and stomach digesta

In the diets, shear stress was positively correlated with WHC (r=0.92, P<0.001) and, consequently, negatively correlated with SR (r=-0.91, P<0.001, **Table 4.6**). In stomach digesta, shear stress positively correlated with the fraction of large particles (r=0.68, P<0.001) and, consequently, negatively with the fraction of middle (r=-0.71, P<0.001) and small particles (r=-0.53, P<0.001). Additionally, in stomach digesta, shear stress was positively correlated with WHC (r=0.41, P<0.001) and negatively with SR (r=-0.76, P<0.001).

All three volume fractions of particles in the diets correlated with the pH, but none with the water holding capacity. For the diets, the strongest correlation was identified between the volume percentage of small particles and pH (r=0.90, P=0.001). In stomach digesta, all three volume fractions of particles correlated with the WHC, of which the correlation with middle sized particles was strongest (r=-0.56, P<0.001). All three volume fractions of particles also correlated with the SR, of which the correlation with large particles was strongest (r=-0.58, P<0.001). The pH positively correlated with large (r=0.26, P<0.05) and middle sized particles

(r=0.30, $P<0.05$) and small particles negatively correlated with dry matter content (r=-0.25, $P<0.05$).

Discussion

With the present study we aimed to elucidate the role of digesta rheology in digesta transport through the upper GIT for pigs fed diets widely varying in physical and chemical properties. To this end, we designed nine extreme dietary treatments with varying forms and sources of starch, and measured digesta transport and digesta rheology as well as underlying physical and chemical digesta properties.

Effect of variation in starch form and source on digesta MRT in the upper GIT

Solid fractions of digesta needed on average 4.9 h to pass the stomach and SI of young growing pigs, which is in line with previous research[10,11,39,40]. The effects of digesta passage behaviour on nutrient absorption kinetics were dominated by stomach MRT, as digesta MRT in the stomach was longer than that of the small intestine, which corresponds to previous research[11]. As expected[10,11], we found that the passage rate for the liquid digesta fraction typically exceeded that of solids in the stomach, but not necessarily in the SI.

Our findings indicate that the largest dietary treatment effects on solid digesta MRT were caused by extrusion, which reduced the stomach MRT compared with ground cereals. In addition, diets with extruded barley tended to remain shorter in the SI compared with diets containing ground cereals. This reduction in MRT, caused by processing, is in line with previous research, which reported that hydrothermal treatment decreased the total dry mass in the stomach of pigs[41]. Replacing native starch with gelatinized starch, however, did not decrease gastric retention times in pigs[42], which suggests that the reduction in gastric retention time, caused by extrusion, is related to other feed traits than starch gelatinization.

No differences in MRT of solid digesta in the upper GIT of pigs fed IA and IM were found. This supports previous findings on the glycaemic response of starches with different amylose contents: In this previous study a similar gastric emptying rate was assumed for both low and high amylose diets, which resulted in a strong relation between the *in vitro* digestibility rate and the time of portal glucose appearance *in vivo*[43].

In our study, we observed a shorter MRT of solid digesta in the SI of pigs fed GB compared with pigs fed IB. This difference in MRT originates mainly from SI3 (**Table 4.1**), where the digestion coefficient of starch originating from GB (0.87) was lower than that of IB (0.96)[26]. Consequently, the longer MRT of IB seems caused by other components in the feed matrix than starch, which were mainly soybean meal and hulls in the IB diet. This corresponds well with the reduction in MRT of SI digesta found when replacing soybean with cereal based material[40].

Table 4.6. Pearson correlation coefficients for rheological and physical properties of diets and stomach digesta and MRT of stomach digesta[1].

	Vol% large particles	Vol% middle particles	Vol% small particles	DM	WHC	SR	pH	Stomach MRT_{solid}	Stomach MRT_{liquid}
					Diets				
Shear stress	-0.13	0.23	-0.38	0.78*	0.92***	0.96***	0.66	-0.71*	-0.47
Vol% large particles		-0.97***	-0.81**	-0.03	0.06	-0.24	-0.74*	-0.21	0.28
Vol% middle particles			-0.93***	-0.17	0.05	0.34	-0.83**	0.15	-0.34
Vol% small particles				-0.35	-0.21	-0.46	0.90***	0.03	0.39
DM					0.87**	0.81**	0.50	-0.76*	-0.59
WCH						0.95***	0.44	-0.85**	-0.48
SR							0.66*	-0.76*	-0.55
					Stomach digesta				
Shear stress	0.68***	-0.71***	-0.53***	0.40***	0.41***	0.69***	-0.14	0.33***	0.19
Vol% large particles		-0.92***	-0.88***	0.13	0.51***	0.52***	-0.26*	0.17	0.17
Vol% middle particles			0.64***	-0.05	-0.56***	-0.49***	0.30*	-0.12	-0.12
Vol% small particles				-0.25*	-0.32**	-0.47***	0.16	-0.22	-0.19
DM					-0.37**	0.59***	0.14	0.37***	0.20
WCH						0.53***	-0.20	0.17	0.15
SR							-0.04	0.50***	0.34**

[1]* indicates P<0.05, ** indicates P<0.01, *** indicates P<0.001.

Rheological characterization of diets and digesta

The rheological behaviour of feed and stomach digesta was characterized by their complex moduli; where the storage modulus (G′) indicates elastic, solid-like, behaviour and the loss modulus (G″) indicates viscous, fluid-like, behaviour[44]. For all experimental diets, G′ exceeded G″ and thus tanδ was below 1, which indicates that diets behaved as a weak gel[31,45]. Based on the shear stress, we concluded that isolated and ground diets were easiest to deform. In this study we did not carry out an amplitude sweep prior to the oscillatory frequency sweep. Consequently, we cannot be sure that the frequency sweep was performed in the linear viscoelastic range. Hence, we should take care in the interpretation of the shear stress, which summarises the rheological characteristics of diets and digesta but can reflect both reversible and irreversible viscoelastic behaviour in this study[37].

For all dietary treatments, stomach digesta was characterised as a weak gel, as found previously for stomach digesta of pigs[46]. The low shear stress observed for stomach digesta of pigs fed extruded diets corresponds well with previous research, which report a higher fluidity of stomach digesta for pigs fed hydrothermal treated diets compared to non-hydrothermal treated diets[41]. In our study, shear stress of stomach digesta of pigs fed ground cereals depended on the source of starch included, resulting in a lower digesta shear stress for pigs fed GB, compared with GM and GA.

Upon transport of digesta from the stomach to the SI, the fluidity of digesta increased and rheology measurements as performed for stomach digesta were not possible. The increase in fluidity after passage of the stomach is likely related to the lower DM content in the SI compared to the stomach (on average 13%, data not shown). It is well known that solids are retained longer in the porcine stomach than liquids[10,11], which is consistent with the difference in MRT between stomach liquids and solids, observed in our study. Usually, large particles (diameter > 1-2 mm) remain in the human stomach until the particle size is reduced sufficiently[16,18]. The accumulation of large particles in the stomach will likely have caused SI digesta to consist mainly out of small particles. The apparent viscosity of composite suspensions such as digesta, depends highly on the ratio between the volume fraction of particles and the maximum packing fraction[9]. Due to the lower DM content and smaller, more homogeneous, size of particles in SI digesta, particles present in SI digesta will contribute less to the whole digesta rheology, compared with stomach digesta[15].

Relation between digesta properties and gastric MRT

Confirming our hypothesis, the MRT of digesta in the stomach of pigs can be partly explained by the shear stress of digesta (r=0.33, $P<0.001$, **Table 4.6**). The shear stress is related to all underlying physical properties measured but, surprisingly, does not necessary explain a larger part of variation in MRT than these underlying properties. Especially the SR explains a large fraction of variation in stomach MRT for both solid (r=-0.48, $P<0.001$) and liquid (r=-0.29, $P<0.01$) fractions of digesta (**Table 4.6)**. The SR indicates the digesta water content, as fraction of the theoretical maximum of water that can be held by the dry matter according to its WHC. In

addition to the WHC of digesta, the SR is strongly affected by the total dry mass in the stomach. The total dry mass, in turn, is affected by properties of the insoluble particulate fraction. In the case of liquids, the negative relation between MRT and SR indicates that water held in the digesta matrix is emptied slower from the stomach than free water. This relation appears more complex in the case of solids, as the behaviour of the solid fraction of digesta depends greatly on the properties of the particulate matter. Compared to the diets with ground and extruded cereals, the diets with isolated starches were richer in soybean hulls, soybean meal, and sugar beet pulp[26]. These ingredients generally have higher water holding capacities than maize and barley meals[47]. Based on this higher water holding capacities, we expected a lower SR for stomach digesta of pigs fed diets with isolated starches compared with diets that included ground cereals. The SR of stomach digesta, however, did not differ between diets with isolated starch and diets with ground cereals (**Table 4.5**). To further unravel the relation between the SR and MRT of stomach digesta, we studied Pearson correlation coefficients for digesta properties and MRT after omitting diets with one starch form at a time (data not shown). When omitting diets with ground cereals from the data set, we observed an increase in the relation between digesta WHC and SR (r=-0.75, $P<0.001$) whilst the relation between digesta SR and MRT of solids remained rather constant (r=-0.46, $P<0.001$). This indicates that the decreased SR of digesta of pigs fed diets with isolated cereals, compared with pigs fed extruded cereals, is dominated by the WHC of digesta. In contrast, omitting diets with isolated starch from the dataset resulted in a stronger relation between digesta DM and SR (-0.92, $P<0.001$), but again not in differences in the relation between digesta SR and MRT of solids (r=-0.54, $P<0.001$). This indicates that the decreased SR of digesta of pigs fed diets with ground cereals, compared with pigs fed extruded cereals, is dominated by the DM content. The DM content in the stomach of pigs fed ground cereals, was higher than that of pigs fed diets with isolated starch, whereas the total weight of stomach digesta did not differ between those dietary treatments ($P>0.1$, data not shown). It seems that more solid particles accumulate in the stomach of pigs fed ground cereals, than in those of pigs fed diets with isolated starches. In conclusion, a considerable part of the variation in gastric MRT can be explained by the SR of digesta, which appears to depend greatly on the physical properties of the particulate matter in the stomach.

Predicting gastric MRT with dietary characteristics

In contrast to the negative correlation between digesta SR and MRT of solids in the stomach of pigs, dietary SR correlated positively with MRT. Dietary WHC was especially high, causing a low dietary SR, in diets containing extruded cereals, particularly barley and maize. This increase in WHC is caused by starch gelatinization during extrusion, which greatly increases the WHC of starch[48-50]. HA maize starch has, due to its molecular properties, a higher gelatinization temperature, which results in a lower degree of gelatinization compared with barley and maize when extruded under similar conditions[49,51,52]. The physiological function of the stomach, however, causes several changes in physical and chemical properties of

diets compared with digesta. This led to different relations between (1) WHC and SR and (2) properties of the particulate fraction and SR, for diets and stomach digesta. Firstly, the strong correlation observed between dietary WHC and SR (r=-0.88, $P<0.001$), was much lower for stomach digesta (r=-0.48, $P<0.0001$). Using chromatographic analysis, we observed break down products of starch upon ingestion, predominantly in extruded diets. Breakdown of the starchy network may explain the decrease in WHC from diets to digesta, and consequently the increase in SR. This fits well with previous research reporting a higher fluidity of stomach digesta in pigs fed hydrothermal processed diets compared with pigs fed unprocessed diets[41]. Starch breakdown in the stomach may also explain earlier observations of a starch-induced increase in dietary WHC, which led, unexpectedly, not to an increased stomach MRT of solids[42]. Secondly, the volume percentage of large particles in the stomach correlated negatively with SR (r=-0.58, $P<0.0001$), whereas this correlation was absent in the diets. Large particles constituted a greater volume fraction of stomach digesta than in the diets, which complicates the prediction of the contribution of the particulate matter to whole digesta properties and rheology. In turn, both the accumulation of large particles and the decrease in WHC during retention in the stomach, hinder predictability of gastric retention times based on feed properties.

Conclusions

The greatest effects of dietary treatments on solid digesta MRT of pigs fed starch rich diets were observed in the stomach, where extrusion reduced MRT of solids by 29 to 75 min. Rheological analysis of whole digesta revealed that gastric digesta behaved as a gel-like material. Variations in digesta shear stress explained part of the variation in solid stomach digesta MRT, but not in liquid digesta MRT. Relationships among rheological properties and small intestinal MRT were absent. Unexpectedly, not shear stress, but the newly introduced parameter saturation ratio (SR) explained most variation in stomach MRT of both solids and liquids: An increased SR related to a decreased MRT. The low SR of extruded diets, related to the high WHC of gelatinised starch, increased considerably after ingestion. Large particles accumulated in the stomach of pigs and correlated negatively to the SR of stomach digesta, but not to that of diets. Due to these changes in chemical and physical properties upon ingestion, the MRT of stomach digesta cannot be easily predicted from dietary properties.

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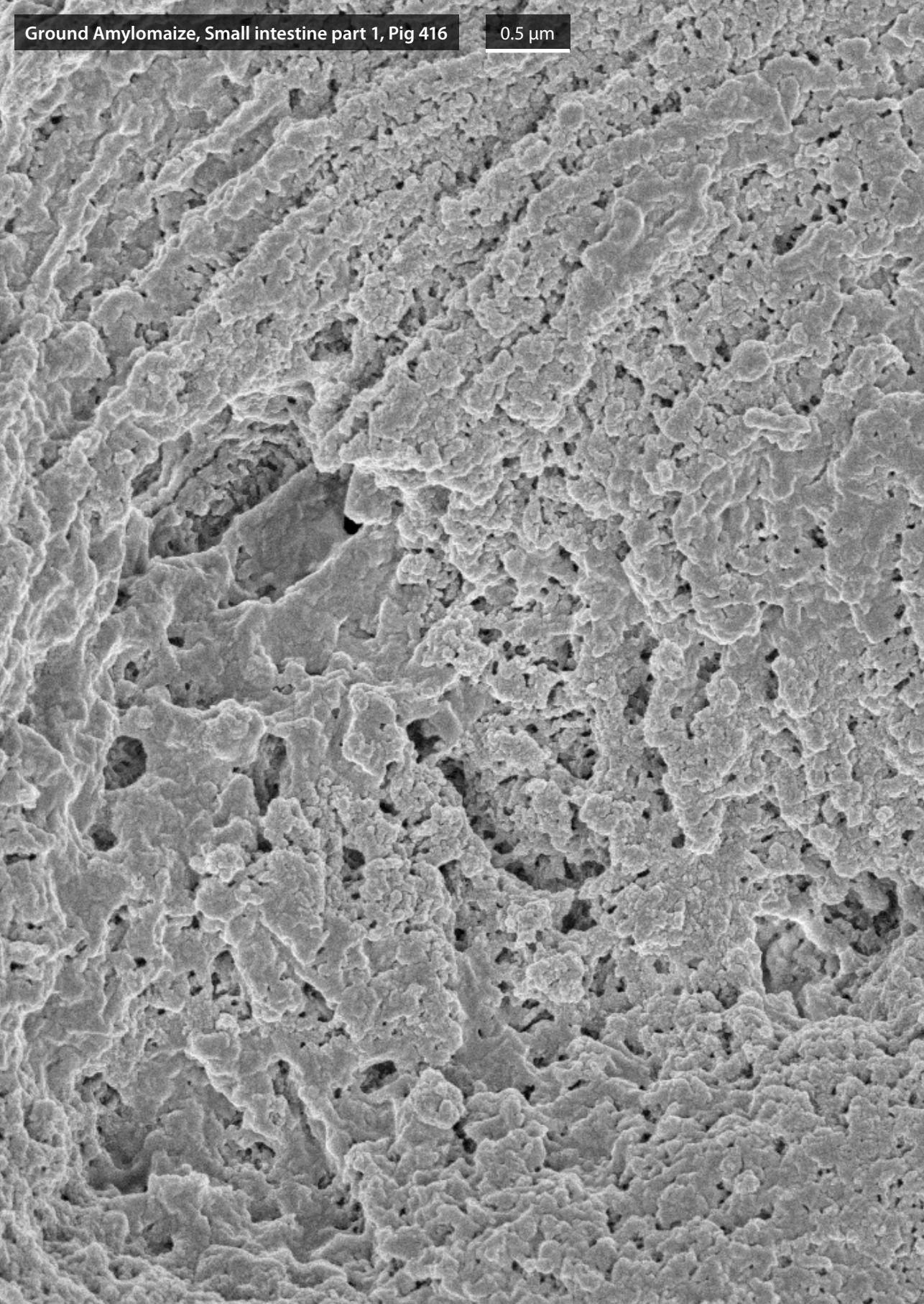
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Chapter 5

Gastric emptying patterns in pigs as measured with the ^{13}C breath test

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To be submitted

Abstract

The ^{13}C breath test is a commonly used method to study gastric emptying of humans, but has not found its way yet to the domain of animal science. In this study, we used the breath test to measure gastric emptying in young growing pigs using ^{13}C octanoic acid to monitor gastric emptying of digesta solids and ^{13}C glycine to study liquids. Pigs were fed a starch rich diet, varying in starch source (isolated starch from barley, maize, or high amylose maize) or form (isolated barley starch, ground barley, or extruded barley), after which $^{13}CO_2$ recovery was measured at several time points during 11 hours. In general, pigs were easy to train and the tailor-made mask allowed effortless sampling. Gastric emptying of all pigs followed a biphasic pattern, with a higher ^{13}C recovery during the first peak. The first peak in gastric emptying of solids reached its maximum atom percentage enrichment within two hours after feeding in all cases. For digesta liquids, this peak was reached earlier for pigs fed ground barley (2.2 hours after feeding), compared to pigs fed diets containing isolated starch (2.8 hours after feeding). The second peak in gastric emptying of solids was reached later for pigs fed ground barley (5.9 hours after feeding), compared with pigs fed extruded barley (4.5 hours after feeding) and pigs fed diets containing isolated barley starch (4.8 hours after feeding). In conclusion, the ^{13}C breath test is a convenient, non-invasive tool to gain more insight in the gastric emptying pattern of pigs.

Introduction

The rate of nutrient absorption in the gastrointestinal tract (GIT) of pigs is known to affect pig performance[1,2]. In turn, nutrient hydrolysis rate and digesta transport are important determinants of nutrient absorption. Due to the long retention time of digesta in the stomach, relative to their residence in the small intestine, stomach emptying plays a major role in nutrient absorption kinetics[3]. Gastric emptying is, amongst others, affected by rheological and physical properties of digesta. For example, an increased fraction of large particles[4] and a highly viscous liquid phase[5] reduces solid digesta passage rates. Digesta properties are determined by the dietary composition and processing. Typically, pig diets consist for 40-50% of starch[6], which makes starch an important contributor to digesta properties. Previously we observed that extrusion of starch rich diets decreased gastric retention times in pigs, using the slaughter method[7].

Although not widely used in animal studies, a frequently used method to measure gastric emptying in human intervention studies is the ^{13}C breath test. The ^{13}C breath test is a minimally invasive technique with broad applications, such as nutrient digestion, digesta transport, oxidation processes, and enzymatic activities, which depend on the choice of ^{13}C-labelled substrate used[8]. For example, the ^{13}C breath test can be used to study gastric emptying when a tracer is used that is not digested in the stomach and rapidly absorbed in the duodenum, such as labelled octanoic acid and glycine[9]. The breath test is generally considered a valid method to study gastric emptying in humans[10]. Firstly, because gastric emptying measured by humans with the ^{13}C breath test have a high degree of reproducibility within individuals[11]. Secondly, because similar rankings for gastric emptying rates have been reported between the breath test and scintigraphy measurements[9,10,12] and between the breath test and magnetic resonance imaging (MRI) measurements[13]. Nevertheless, the physiological meaning of breath test parameters is still debated upon. Discrepancies in gastric half time are reported between those calculated from breath test parameters and those observed in real emptying processes, e.g. when measured by MRI[13]. These discrepancies are, amongst others, caused by incomplete retention of $^{13}CO_2$ and delayed recovery due to sequestration of $^{13}CO_2$ by various organs[10].

In the present study we assessed gastric emptying of both liquid and solid digesta fractions for pigs fed a meal varying in starch form and source, using the non-invasive ^{13}C breath test. With this test, we aimed to gain more insights in the patterns of gastric emptying after meal feeding. We hypothesized that pigs, due to their curious nature, would be easily trained to breath voluntarily through a mask, which would allow for non-invasive gastric emptying measurements.

MATERIAL AND METHODS

The experiment described in the present manuscript was part of a larger study on starch digestion kinetics and digesta passage behaviour, which is described in detail elsewhere[7]. The experiment was approved by the Dutch Central Committee of Animal Experiments under the authorization number AVD260002016550.

Experimental design, animals, and diets

Briefly, 90 crossbred gilts (Topigs 20 × Pietrain sire), weighing 23.1 ± 2.1 kg, were assigned to one of nine dietary treatments in a 3 x 3 factorial arrangement, in four successive batches. Factors were starch source (barley, maize, high amylose (HA) maize) and form (isolated starch, ground cereal, extruded cereal). The experiment consisted of an adaptation period of at least 2 days, followed by an experimental period during which the experimental diets were fed at 2.0 × the energy requirements for maintenance (750 kJ NE per kg BW^0.60)[14]. The experimental diets were fed in two equal meals at 8:00 and 16:00, for at least 5 days. All the diets were mixed with water just before feeding, to stimulate feed intake and to prevent sedimentation. Diets were formulated to meet or exceed the nutrient requirements of growing pigs[14], designed to contain ~400 g starch per kg dry feed, and to be identical in protein, fat, and NSP content. Details on ingredients, feed production conditions, the analysed dietary composition, and rheological and physical properties are described elsewhere[7].

Breath sampling and analyses

Gastric emptying patterns were measured on two consecutive days by breath sample analysis. On the days of the breath test, pigs received a morning meal which met 0.9 × maintenance energy requirement (750 kJ NE per kg BW^0.60)[14]. The meals were enriched with 86±1.6 mg of either [1-^{13}C]- glycine or [1-^{13}C]- octanoic acid (Cambridge isotope laboratories, Andover, USA), to study gastric emptying of respectively the liquid or solid fraction of the meal[9]. The water soluble glycine was mixed through an aliquot of water, prior to mixing the water with the dry feeds. Octanoic acid was dissolved in 50 ml 96% ethanol, which was mixed trough the dry feed on the day before the breath test, allowing the ethanol to evaporate overnight. Wet feeds were prepared directly before feeding. Pigs were fed individually, with a 5 min interval between every 4 pigs, creating a time frame of 5 min for the breath sample collection of 4 pigs. Samples were collected at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 9, and 11 hours after the morning meal.

Pigs were trained via a reward system, using small treats such as jelly beans, to voluntarily breathe through a mask that was tailor-made in house. This mask consisted of a macrolon narcotic mask (**Figure 5.1,** item **1**) (size 6, Henry Schein Animal Health, Cuijk, the Netherlands) with a rubber cuff (**Figure 5.1**, item **2**) (Henry Schein Animal Health), which guaranteed that the mask fitted air tight around the snout of the pig. The airflow in the mask was controlled with several one-way valves (Intersurgical Benelux B.V., Uden, the Netherlands), in such a

way that fresh air entered at the top of the mask via one inlet valve (item **3**) and breath left the mask via two outlet valves (items **4**). Consequently, a small chamber (~15 ml) was created (item **5**), containing breath that was in direct contact with a needle (item **6**), to which a vacuum glass vial of 5.9 ml with a pierceable septa (Soda Glass Vials, Labco, Lampeter, UK) could be connected. The vacuum inside the vial caused breath to be drawn in the vial, which allowed for rapid sampling that did not hinder the animals. Tubes were stored at 4°C until analysis.

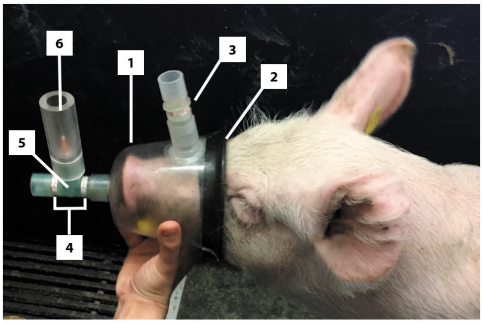

Figure 5.1. Breath sample collection via a tailor-made mask, consisting of a macrolon narcotic mask (item **1**), of which an air tight fit was secured by a rubber cuff (item **2**). Fresh air entered at the top of the mask via one inlet valve (item **3**) and breath left the mask via two outlet valves (items **4**). Consequently, a small chamber (~15 ml) was created (item **5**), containing breath that was in direct contact with a needle (item **6**), via which the breath could be easily sampled into a vacuum glass vial.

Breath samples were analysed for the ^{13}C enrichment of the CO_2 with a Trace GC Ultra and a stable isotope ratio mass spectrometer in splitless mode (Thermo Fisher Scientific, Massachusetts, USA). The oven of the gas chromatograph was set to 35°C, with a flow of 2.5 mL/min and a split flow of 10 mL/min. The oxidation temperature was set at 940°C and the reduction temperature at 650°C. Argon was used to flush the air within the gas chromatograph between the measuring of different samples and each sample was analysed in duplicate.

Statistics and calculations

^{13}C-tracer recovery was expressed at each time point as nett atom percentage enrichment (**APE**) of $^{13}CO_2$, which was calculated by subtracting measured background enrichment values at t=0 from the average atom percentage $^{13}CO_2$ (**AT%**) of duplicate measurements. If the AT% at t=0 was missing or exceeded the AT% at t=0.5, the AT% at t=0.5 was used to calculate the nett APE. Proc NLIN was used to fit a nonlinear regression model based on the derivative of a generalized Michaelis-Menten equation describing a biphasic response, modified from van den Borne et al.[15]:

Equation 5.1.
$$APE = \frac{\left[b_0\alpha \cdot c\alpha \cdot t^{(-c\alpha-1)} \cdot b_1\alpha^{c\alpha}\right]}{\left[1+\left(\frac{b_1\alpha}{t}\right)^{c\alpha}\right]^2} + \frac{\left[b_0\beta \cdot c\beta \cdot t^{(-c\beta-1)} \cdot b_1\beta^{c\beta}\right]}{\left[1+\left(\frac{b_1\beta}{t}\right)^{c\beta}\right]^2}$$

where APE is the nett atom percentage enrichment expressed in percentage, t is the time in hours, and $b_0\alpha$, $b_1\alpha$, $c\alpha$, $b_0\beta$, $b_1\beta$, and $c\beta$ (all between 0 and 10) are parameters that define the curve. The fit of the model was restricted by several bounds, which were based on visual analysis of the data points: (1) the first peak had to appear before the second peak and had to be at least the same size as the second peak. (2) the second peak had to appear before 11

hours, which was the time of the last measurement taken for each animal. From the predicted curves, the time point at which the maximum APE was reached during the first phase, $t_{max\alpha}$, and second phase, $t_{max\beta}$, were calculated as described elsewhere[15]. The gastric emptying pattern was further characterized by the ratio between areas under the curve of both peaks, expressed as the area of peak α as percentage of total area (AUC_{α}) and the area of peak β as percentage of total area (AUC_{β}). Additionally, the ratio in heights of both peaks was expressed by the APE reached at $t_{max\beta}$, as fraction of the APE reached at $t_{max\alpha}$ ($ratio_{\beta/\alpha}$).

During data analysis, inadequate flushing of the chamber (item **5** in **Figure 5.1**) was detected on several occasions, by tracking the observed 13C enrichment to that of the pig previously breathing through the mask. Typically, these points appeared scattered in the response curve, and were identified as follows: (1) A cooks distance outlier test was performed once per animal, in which the APE at each time point was compared with the mean APE of each animal[16]. If cooks distance > 0.07/number of data points per animal, the data point was removed (in total 3.6% of all data point). (2) A leave-one-out analysis (LOOA) was performed in which the fit of **Equation 5.1** through all data points was compared with the fit of **Equation 5.1** when one data point was left out. If the SSE decreased by at least a factor ten when leaving a data point out, this data point was deleted. The leave-one-out analysis was repeated until the removal of data points did not decrease the SSE with at least a factor ten, further. In total, less than 1% of the data points that remained after the cooks distance test was removed based on this analysis. If less than four data points remained in the first three hours of measurement or less than two in the last four hours, the animal was removed from the database. In addition, pigs that refused >30% of the provided feed, were excluded from breath test measurements. During the experimental period, signs of an *E.Coli* infection were observed for several animals. Some of these animals, mainly animals fed maize or high amylose maize diets in ground or extruded form, were not fully recovered at the moment of breath test analysis. Consequently, five or less than five observations remained after the cooks distance test and LOOA for pigs fed maize or high amylose maize diets in ground and extruding form. Therefore, only gastric emptying patterns of pigs fed barley based diets or pigs fed diets with isolated starches are presented here, which are analysed as two independent datasets.

Effects of experimental treatments on breath test parameters were tested within pigs fed barley based diets or within pigs fed isolated diets, using a general linear model (PROC GLM, SAS, version 9.4, SAS Institute, Cary, USA). For pigs fed barley based diets, starch form and batch were included as class variables and for pigs fed isolated diets, starch source and batch were included as class variables. Least square means were compared after Tukey's adjustment for multiple comparisons. Data are presented as least square (LS) means and standard deviation of the mean (S) unless stated otherwise. Significance was assumed at $P \leq 0.05$, while a tendency was considered when $0.05 < P \leq 0.1$.

Results

In general, pigs could be trained to use the mask within 2 to 3 days. The mask allowed for effortless sample collection and a trained person could generally collect a sample within one minute.

Typical tracer recovery patterns for pigs fed barley based diets are shown in **Figure 5.2**,

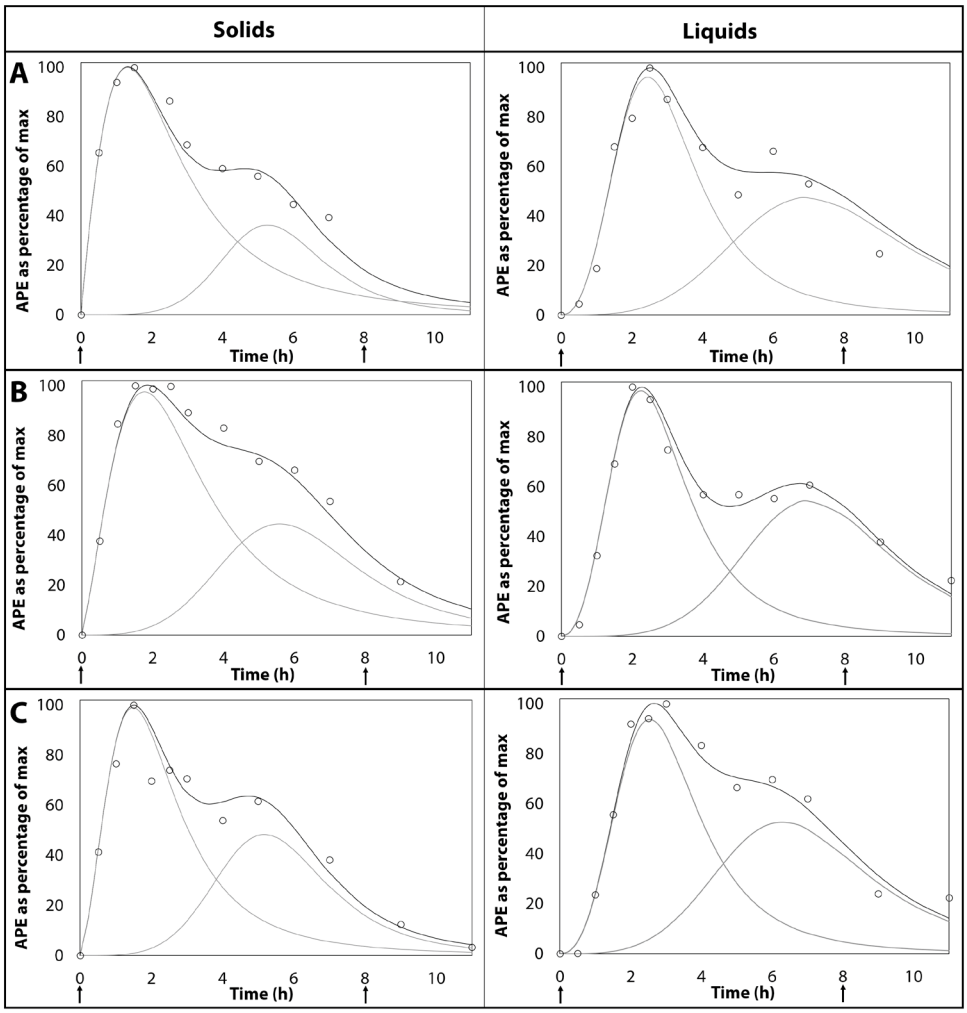

Figure 5.2. Typical gastric emptying curves of solids and liquids, obtained with the breath test method, for pigs fed diets (at time 0) containing isolated barley starch (panel A), ground barley (panel B), or extruded barley (panel C). Gastric emptying is expressed as the atom percentage enrichment (APE) as percentage of max APE measured per pig. The open circles indicate measurement points. The two grey lines in each graph represent the two individual peaks in APE, the black line represents the total APE. The arrows indicates the times of feeding.

Table 5.1. Parameter estimates from a generalized Michaelis-Menten derivative model describing the gastric emptying of pigs fed barley based diets containing starch as isolated powder, ground cereal, or extruded cereal, or diets containing starch as isolated powder, originating from barley, maize, or high amylose (HA) maize.[1,2,3]

	Barley based diets			S	P-value	Diets with isolated starches			S	P-value
	Isolated	Ground	Extruded		Form	Barley	Maize	HA maize		Source
Solids										
Observations	9	9	8			9	7	8		
$t_{max\alpha}$ (Hours)	1.5	1.7	1.6	0.42	0.544	1.6	1.9	1.8	0.57	0.495
$t_{max\beta}$ (Hours)	4.8^{b}	5.9^{a}	4.5^{b}	0.66	**0.001**	5.1	6.2	6.0	1.54	0.292
$Area_{\alpha}$ (%)	59	66	58	11.0	0.278	62	68	58	11.4	0.265
Ratio	0.80	0.67	0.80	0.20	0.307	0.79	0.84	0.90	0.27	0.673
Liquids										
Observations	8	8	8			8	9	6		
$t_{max\alpha}$ (Hours)	2.8^{a}	2.2^{b}	2.7^{ab}	0.39	**0.031**	2.8	2.7	2.7	0.41	0.924
$t_{max\beta}$ (Hours)	7.9	7.4	7.5	0.93	0.431	7.9	8.7	7.9	1.32	0.410
$Area_{\alpha}$ (%)	68	66	61	10.3	0.416	68	62	62	9.63	0.407
Ratio	0.67	0.58	0.64	0.28	0.831	0.67	0.91	0.67	0.30	0.181

[1] Presented values are estimated LSMeans and standard deviation (S). The effect of starch form is analysed within starches of barley origin and the effect of starch source is analysed within diets that contain starch in isolated form.

[2] Model established p-values for fixed effects of starch form (isolated, ground, or extruded) analysed within pigs fed barley based diets and fixed effects of starch source (barley, maize, or high amylose (HA) maize) analysed within pigs fed diets containing isolated starch. Superscripts ^{a,b} indicate differences among treatments ($p<0.05$).

[3] $t_{max\alpha}$ is the time point after feeding at which the maximum atom percentage enrichment (APE) in ^{13}C of the first gastric emptying phase is reached, whereas $t_{max\beta}$ represents the time to reach the peak of the second phase. $Area_{\alpha}$ represents the area under the curve of peak α, as percentage of the area under the total curve.

illustrating a biphasic gastric emptying pattern for all forms of barley starch fed. ^{13}C recovery originating from octanoic acid, associated with the solid digesta fraction, reached a first peak ($t_{max\alpha}$) within 2 hours after feeding for all treatments (**Figure 5.2**, **Table 5.1**). ^{13}C originating from glycine, associated with the liquid digesta fraction, reached a first peak within three hours after feeding. For digesta liquids, $t_{max\alpha}$ was reached 0.6 hours later for pigs fed ground barley, compared with pigs fed isolated barley starch (P<0.05). In addition, $t_{max\alpha}$ of digesta liquids tended to be reached 0.5 hours earlier for pigs fed ground barley, compared pairwise with pigs fed extruded barley (P<0.1). Peak α was followed by a second peak (peak β), which maximum recovery ($t_{max\beta}$) was reached 4 - 7 hours after feeding for the solid digesta fraction and 7 - 9 hours after feeding for the liquid digesta fraction. For digesta solids, $t_{max\beta}$ was reached 1.1 - 1.4 hours later for pigs fed ground barley, compared with pigs fed diets containing isolated barley starch and diets containing extruded barley (P<0.001). For pigs fed diets containing isolated starches, the source of starch did not affect $t_{max\alpha}$ nor $t_{max\beta}$, for both digesta solids and liquids. The area under the curve of peak α (AUC_{α}) was not affected by treatments and was on average 62% of the total AUC for digesta solids and 65% for digesta liquids. Also the ratio between the heights of peak α and β ($ratio_{\beta/\alpha}$) did not differ between treatments for both liquid and solid digesta. Across treatments, $ratio_{\beta/\alpha}$ averaged 0.69 for digesta liquids and 0.80 for digesta solids.

Discussion

In this study, we observed a biphasic gastric emptying in young growing pigs using the ^{13}C breath test method, which contrast earlier work that describes a single phase gastric emptying in humans[17] and pigs[1] using a similar breath test method. To compare the gastric emptying rate of solids measured in the present study with previous studies, we estimated an average time to peak (average t_{max}) in gastric emptying of solids, according to the following equation:

Equation 5.2.

$$average\ t_{max} = t_{max\alpha} * \frac{area_{\alpha}}{100} + t_{max\beta} * \frac{area_{\beta}}{100}$$

The estimated average t_{max} for digesta solids did not differ between treatments and was 3.1 h across treatments, which is in line with values reported previously for gastric emptying of solids in humans (0.8 to 6.0 hours)[17] and sows (1.5 to 6.0 hours)[1].

We found that, for digesta solids, the second peak in gastric emptying appeared later for pigs fed ground barley compared with pigs fed diets containing isolated barley starch or extruded barley. This fits well with our previous study, in which the gastric mean retention time (MRT) for digesta of pigs fed ground cereals was higher than extruded cereals (P<0.05) and numerically higher than diets with isolated starch[7]. In the current study, the first peak in gastric emptying of liquids appeared faster for pigs fed ground barley compared to pigs fed diets containing isolated barley, which does not correspond well with the MRT as we measured previously[7]. In our previous study, we found that solid MRT in the porcine stomach ranged from 129 to 225. Solid digesta of pigs fed extruded cereals remained 29 to 75 min shorter in the stomach

compared with pigs fed ground cereals (P<0.001). Solid MRT consistently exceeded that of liquids in the stomach, but was not affected by dietary treatments. In the previous study, pigs were fed small, hourly portions in order to reach a steady state, in which digesta flows with a continuous rate from one compartment to the next. In this study, however, the gastric emptying was measured after pigs were fed a meal that was around trice the size of the hourly portion. This discrepancy in initial gastric emptying rates is likely related to other factors in addition to the physicochemical properties of digesta, such as the meal size[18] and total caloric content[19].

Methodological considerations

Obviously, any indirect measurement of digesta flow, contrary to direct measurements such as MRI, depend on the suitability of tracer used. In the basics, both tracers used in this study are rapidly absorbed in the duodenum and oxidised in the liver, and both carry a 1-^{13}C, which is cleaved off during oxidation and subsequently party exhaled[8,12]. Consequently, the rate at which both molecules leave the stomach is limiting for the appearance of $^{13}CO_2$ in the breath. Despite being amongst the first tracers used to study gastric emptying by Maes et al. in 1994[9], and being used in gastric emptying measurements ever since[8,11,12], the exact behaviour of both molecules in the complex digesta matrix is not fully understood yet. The solubility of glycine is much higher (over 200 g/L) than that of octanoic acid (~0.85 g/L) in pure water of 39°C[20]. Consequently, glycine included as tracer in a breath test analysis is assumed to be good tracer for the liquid fraction of digesta. Octanoic acid, in contrast, is assumed a good tracer for the solid fraction of digesta. Its behaviour, however, is strongly affected by the presence of other nutrients in digesta, especially fat. For example, an unstable lipid emulsion caused a 98 minute delay in $^{13}CO_2$ appearance from ^{13}C-octanoic acid compared to a stable lipid emulsion[21]. In addition, ^{13}C appeared earlier in the breath of test subjects when an egg yolk, containing ^{13}C octanoic acid, was prepared separately from egg white[11] compared to when it was prepared as scrambled egg[17].

Gastric emptying of solids versus liquids

In this study, we found an unexpected faster stomach emptying of octanoic acid than of glycine. We assume to have a negligible delay in $^{13}CO_2$ appearance from octanoic acid caused by dietary fat, due to the low level of fat (~6%) and high homogeneity of diets, caused by the thoroughly mixing before feeding, compared to previous studies[11,21]. Consequently, we suspect that octanoic acid indeed followed digesta solids and that glycine followed digesta liquids. It seems that the behaviour of the tracers alone does not explain the unexpected difference identified between gastric emptying of solids and liquids. The difference seems to originate from the lag-phase observed during the initial emptying of digesta liquids, which was absent during emptying of digesta solids (**Figure 5.2**). Based on previous research, this lag-phase was expected for solids, but not for liquids[22]. The lag-phase in gastric emptying of solids is usually caused by particles that need time to be degraded to a size that is small

enough to leave the stomach[22], which is generally ~1-2 mm. On average, the solid fraction of dietary treatments used in this study consisted for 88 vol% of particles smaller than 1 mm (calculated from data presented elsewhere[7]). This suggests that a major fraction of the digesta solids is not hampered in immediate stomach emptying, which would have led to the absence of a lag-phase during gastric emptying of solids. The presence of a lag-phase upon emptying of liquids might be related to the high water holding capacity of the diets used in this trial[7], encapsulating glycine in the digesta matrix and thereby delaying gastric emptying. An unambiguous explanation, however, was not found in this study.

Conclusions

We conclude that the ^{13}C-breath test is a convenient, non-invasive alternative to measure digesta passage behaviour in pigs, although the ranking in gastric emptying of solids and liquids differed from our expectations. Animals were easy to train, which allowed for rapid sample collection. Upon ingestion of starch rich meals, we identified a biphasic gastric emptying pattern. Pigs fed ground barley had a delay of approximately 1 hour in the second phase of gastric emptying of solids, compared with other forms of barley starch fed. For digesta liquids, the first peak of gastric emptying was reached earlier for pigs fed ground barley compared to pigs fed diets containing isolated barley starch. Amongst pigs fed diets containing isolated starch, the source of starch did not affect gastric emptying patterns.

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Extruded Maize, Small intestine part 1, Pig 423
5 µm

Chapter 6

The importance of gastric amylases in starch digestion kinetics in pigs

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Submitted for publication

Abstract

Starch digestion in the proximal small intestine of pigs exceeds *in vitro* predictions, suggesting a currently underestimated role for the stomach in starch digestion kinetics. This study aimed to investigate the role of gastric amylases on starch digestion in pigs, including hydrolysis by porcine saliva and degradation by bacterial enzymes present in the stomach. We studied the hydrolysis of starch in pigs fed barley based diets, in which starch was included as isolated powder, ground barley, or extruded barley. We identified soluble maltodextrins originating from starch break down in stomach digesta, especially in pigs fed extruded barley. Evidence of bacterial degradation of granular starch was observed with electron microscopy, for pigs fed diets containing isolated barley starch or ground barley. These observations lead us to measure starch hydrolysis in a dynamic stomach model over the course of a 225 min incubation, in which the pH was step-wise decreased from 6.5 to 2.0. Using this method, feed was either exposed to an enzyme extract obtained from stomach digesta or to porcine saliva. Up to 29% of starch was hydrolysed into maltodextrins with a degree of polymerisation (DP) <6 when starch was incubated with the enzyme extract obtained from the stomach. Under the same conditions, saliva amylase, with an optimum pH around 7.8, hydrolysed up to 10% of processed starch into maltodextrins with DP<6. We conclude that a substantial part of starch may be degraded into oligomers in the porcine stomach, whereby starch hydrolysis by bacterial amylases overrules starch hydrolysis by salivary amylases.

Introduction

For most starches, the vast majority of starch disappearance in pigs occurs in the proximal small intestine. Digestion coefficients (DC) of starch in the proximal small intestine reached values of 0.45 for barley meal[1], 0.64 for oat meal[2], 0.60[3]-0.72[1] for maize meal, and 0.75 for wheat meal[1]. Recently, we measured starch disappearance and digesta retention times throughout the small intestine of pigs, aiming to compare *in vivo* and *in vitro* starch hydrolysis kinetics. Across the nine dietary treatments used in our study, *in vitro* and *in vivo* measurements differed considerably: On average 35% of starch disappeared from the small intestine within 5 minutes, whereas only 14% of starch was digested during the same time of *in vitro* incubation[4]. Consequently we considered that, *in vivo*, starch may be partly degraded in the stomach. This theory fits well with previous research, where almost half of the ingested starch appeared to be hydrolysed and absorbed proximal of the pancreatic duct[5].

Several digestive processes may play a role in gastric digestion of starch in pigs, for example hydrolysis by porcine saliva or by gastric bacteria. For a long time, it is believed that salivary α-amylase does not quantitatively contribute to starch degradation[6,7], as the pH in the porcine stomach ranges typically from 2-4[8]. In a more recent study however, pH values up to 6.9 were measured in the proximal region of the porcine stomach 20 min after feeding, which remained above 5 up to 180 min after feeding[9]. Human salivary amylase is active at pH values between 3.8 and 9.4[10], which suggests that salivary α-amylase could act upon starch for a prolonged time after ingestion. Indeed, a recent study revealed that up to 80% of starch is hydrolysed *in vitro* by human salivary α-amylase in 90 min, when the pH was gradually decreased from 6 to 2[11]. Whether this extent of starch degradation under comparable stomach conditions is also the case for porcine salivary amylase is not sure, as much less is known about this enzyme. Next to saliva amylase, also bacterial amylases may contribute to starch degradation in the porcine stomach. Considerable amounts of bacteria, up to 10^9 viable cells per gram digesta have been reported in the porcine stomach[12,13], as well as the production of short chain fatty acids[8,14,15]. However, the extent to which starch is hydrolysed in the porcine stomach has not yet been reported.

Partial starch breakdown in the stomach of pigs seems plausible, but the quantitative contribution of both salivary and bacterial amylases remains unknown. We hypothesise that a considerable part of starch can be degraded in the stomach of pigs. To this end, we performed two studies. In the first study, we assessed the *in vivo* concentration and structures of unabsorbed starch residuals in the porcine stomach. Furthermore, in the second study, we studied the fate of starch in an dynamic *in vitro* stomach model, in which the pH was gradually decreased from 6.5 to 2.0 and feed was incubated with enzymes extracted from stomach digesta of pigs. Additionally, we measured porcine saliva characteristics and its potential to degrade starch under dynamic stomach conditions.

Materials and methods

In this manuscript, two studies are described. Study 1 focused on starch degradation products in the stomach of pigs and was part of a larger study performed by our group, which focussed on starch digestion kinetics in the small intestine and is described in detail elsewhere[4]. The experiment was approved by the Dutch Central Committee of Animal Experiments under the authorization number AVD260002016550. Study 2 focused on *in vitro* simulations of starch degradation in the stomach, using porcine salivary amylases or enzyme extracts obtained from the pigs in study 1.

Study 1: Unabsorbed breakdown products of starch in the porcine stomach

Experimental design, Animals, and diets

Briefly, 30 crossbred gilts (Topigs 20 × Pietrain sire), weighing 23.2 ± 1.3 kg, were assigned to one of three dietary treatments. Diets contained only starch from barley origin, which was included as isolated barley starch (IB), ground barley (GB), and extruded barley (EB). Pigs were fed the experimental diets for at least 12 days and each pig was fed individually at 2.0 × the energy requirements for maintenance (750 kJ NE per kg BW^0.60)[16]. All the diets were mixed with water just before feeding, to stimulate feed intake and to prevent sedimentation. During the last two days of the experimental period, the daily allowance of the pigs was equally divided over multiple meals a day, in an attempt to maintain a constant filling and digesta passage rate through the gastro-intestinal tract (GIT). Diets were formulated to meet or exceed the nutrient requirements of growing pigs[16] and designed to contain ~400 g starch per kg dry feed and to be identical in protein, fat and NSP content. Details on ingredients and analysed dry matter, starch, nitrogen, crude fat, ash, and NSP contents are described elsewhere[4]. Chrome (Cr) and Cobalt (Co) were included as markers in the feed at a level of 170 mg/kg (w/w, as fed basis), in the form of chromium oxide (Cr2O3) and Co-EDTA, respectively.
Prior to dissection, pigs were sedated and exsanguinated. Immediately after exsanguination, a clamp was placed between the stomach and small intestine to prevent the movement of digesta and the stomach was carefully removed. The stomach content was homogenised by manual mixing prior to the collection of two digesta samples: One was immediately frozen and kept at –20°C, the other was frozen 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 12 000 r.p.m. (ZM200; Retsch, Haan, Germany). All enzymes and chemicals used for further analysis were purchased from Merck KGaA (Darmstadt, Germany) unless stated otherwise.

Analysis of unabsorbed starch residues in stomach digesta

The concentrations of free glucose and soluble maltodextrins were analyzed in duplicate in freeze dried digesta with a High Performance Anion Exchange Chromatography system with Pulsed Amperometric Detection (HPAEC-PAD) as described previously[4]. Equal aliquots (weight based) of digesta samples were pooled across all pigs within a dietary treatment.

The structure of unabsorbed starch residuals in porcine stomach digesta was analyzed with a Scanning Electron Microscope (SEM) as described previously[4]. Briefly, one pig per treatment was selected that was shown to have digesta mean retention times (MRT) and starch DC in the stomach and small intestinal compartments that were close to the average MRT and DC within that treatment. Digesta of this pig, directly frozen after collection, was washed subsequently with hexane, twice with demi water, and finally with 96% ethanol. Following, the dried digesta was attached on SEM sample holders and analyzed with a field emission SEM (Magellan 400, FEI, Eindhoven, the Netherlands) with secondary electron (SE) detection at 2 kV.

Study 2: *In vitro* simulations of starch degradation in the stomach of pigs

Extraction of enzymes from porcine stomach digesta

We created a representative enzyme extract from porcine stomach digesta by pooling digesta of one pig for each treatment across all nine dietary treatments that were used in the original animal trial[4]. Pigs were selected that had mean retention times (MRT) and starch DC in the small intestinal compartments that were close to the average MRT and DC within that treatment. Ten grams of frozen digesta per pig were thawed and digesta from all pigs were pooled together. Pooled digesta were stirred on ice for 1 hour at pH 5 in a 50 mM sodium acetate buffer containing 2 M NaCl. Subsequently, bacterial cells in the digesta were lysed by sonication (Sonifier S-250D, Branson, Danbury, CT, USA) in 4 sets of 60 s at an amplitude of 100%, during which the pulse was alternatively turned on and off for periods of 10 s. Supernatant containing lysed cells were centrifuged twice for 22.5 min at 4°C and 48 000 *g*, after which the supernatant was filtered through a 0.45 µm Minisart® NML syringe filter (Sartorius, Göttingen, Germany) to remove undamaged bacterial cells and small feed particles. Subsequently, the obtained enzyme cocktail was concentrated and desalted at 4°C, using Amicon Ultra-15 10K centrifugal filter devices (Merck Millipore, Billerica, MA, USA). To this end, the volume of the enzyme cocktail was reduced approximately ten times and subsequently diluted approximately ten times, using a 50 mM sodium acetate buffer at pH 5. This procedure was repeated twice, except for the final dilution step. The concentrated enzyme solutions were stored at -20°C until analysis.

Collection of porcine saliva

Saliva was collected from a mixed group of 48 crossbred gilts and boars (Topigs 20 × Pietrain sire), BW 20-25 kg, which were housed with six animals per pen and belonged to the experimental farm herd of Agrifirm Innovation Center (Laverdonk, Heeswijk-Dinther, The Netherlands). Twenty-four cotton ropes, cut and fastened to the pens to reach shoulder height of the pigs, were divided equally amongst the pens. Pigs were allowed to chew on the ropes for 15 minutes, after which the saliva was collected. The recovered saliva was pooled and frozen at -20°C. Prior to analysis, each aliquot of saliva was centrifuged for 10 min at 2000 g to remove insoluble particles.

In vitro starch hydrolysis under dynamic stomach conditions

Three *in vitro* incubations were performed with each diet to quantify starch hydrolysis under dynamic stomach conditions. One incubation was performed without addition of starch hydrolysing enzymes (referred to as blank incubation), one with addition of enzymes extracted from stomach digesta, and one with addition of porcine saliva. For the blank incubation, 1 g of feed was mixed with 25 mL demineralised water and 65 mg porcine pepsin (P-7000), and stirred at 39°C for 15 min. Subsequently, using a pH-stat device (Metrohm, Herisau, Switzerland), the pH was decreased from pH 6.5 to pH 2 with 0.5 M HCl in nine steps of 0.5 pH units and 22.5 minutes each, creating a total incubation time of 225 min. A typical pH distribution over time is visualised in **Figure 6.1**. The incubations with starch hydrolysing enzymes had a similar pH gradient and incubation times. For the incubation with enzymes extracted from stomach digesta, 2 mL of extract was added just before the pH was lowered to 6.5. This ratio equals the observed ratio of stomach fluids to starch, which was measured in study 1 (data not shown). For the incubation with saliva, 1 mL of saliva was added to the suspension just before the pH was lowered to 6.5. For all incubations, samples were taken prior to the addition of enzymes and prior to every pH step and samples were directly diluted 10 times with demineralised water of 99°C. After 10 min at this temperature, the diluted samples were cooled to room temperature, centrifuged for 10 min at 2000 g, and glucose and maltodextrins levels were analysed with HPAEC-PAD, as described previously[4].

Characterisation of porcine salivary amylase

Dry matter (DM) content of the saliva was determined by drying an aliquot of saliva at 60°C until the weight remained stable. Following, the total nitrogen content was determined by the Dumas method using Flash EA 1112 NC analyser (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol using methionine as standard. The nitrogen content was multiplied by 6.25 to obtain the protein content.

The pH optimum of salivary amylase was determined by measuring the amylase activity at pH values between 3 and 8.5, according to the alpha-amylase assay kit from Megazyme (Wicklow, Ireland), with slight adjustments. Briefly, salivary amylase was incubated with non-reducing-end blocked p-nitrophenyl maltoheptaoside for 30 min at 39°C, after which the pH was adjusted to 6.0 and the solution was heated to stop α-amylase activity. Following, formed p-nitrophenyl maltosaccharides were degraded by α-glucosidase and the concentrations of released p-nitrophenyl were measured spectrometrically.

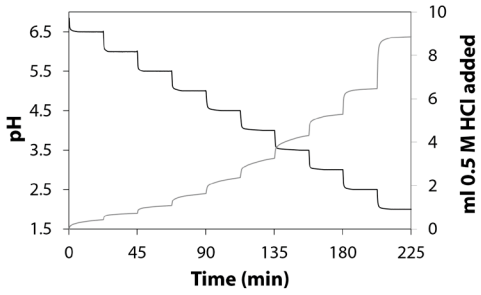

Figure 6.1. ml of 0.5 M HCl added (grey line, secondary x-axis) to 1 gram feed in 25 mL water over time, and resulting pH gradient (black line, primary x-axis), of the *in vitro* dynamic stomach model used.

To measure the activity of porcine salivary amylase on starch, 0.3 mL of saliva was incubated at 39°C at its optimum pH, with either 50 mg native maize starch (Roquette, Lestrem, France) or 50 mg pregelatinized potato starch (*Paselli*, Avebe, Veendam, the Netherlands). After 30 min, the mixture was boiled for 10 min and centrifuged. Starch breakdown was quantified as the number of reducing sugars formed per min per mg saliva DM, which was measured with the PAHBAH reducing sugar assay[17]. Briefly, 10 µl of the supernatant of the incubated starch solution was mixed with 200 µl of PAHBAH reagent, containing 2 µg 4-hydroxybenzoic acid hydrazide. After 35 min incubation at 70°C, reducing end formation was measured spectrophotometrically. In addition, pancreatic amylase was measured as reference, by incubating porcine pancreatin (P-7545) for 15 min, using the same procedure as described for salivary amylase.

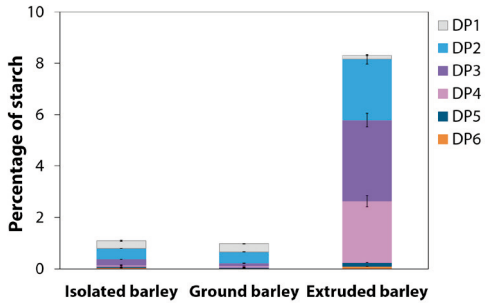

Figure 6.2. Percentage of starch solubilized in the stomach of pigs fed diets containing barley starch included as isolated powder (I), ground cereal (G), or extruded cereal (E). The solubilized starch is divided into individual maltodextrins up to degree of polymerisation (DP) 6, and soluble maltodextrins with a DP>6. The error bars represent the standard error of measured maltodextrins concentrations.

Results

Study 1: Unabsorbed starch in the stomach of pigs

The concentration of soluble maltodextrins in the stomach increased upon ingestion of all diets, especially in pigs fed extruded barley. For pigs fed extruded barley, soluble starch breakdown products in stomach digesta consisted mainly of maltose, maltotriose, and maltotetraose (**Figure 6.2**). For the other treatments, the sum of concentrations of breakdown products measured was on average 7.3 percentage point lower and consisted mainly of

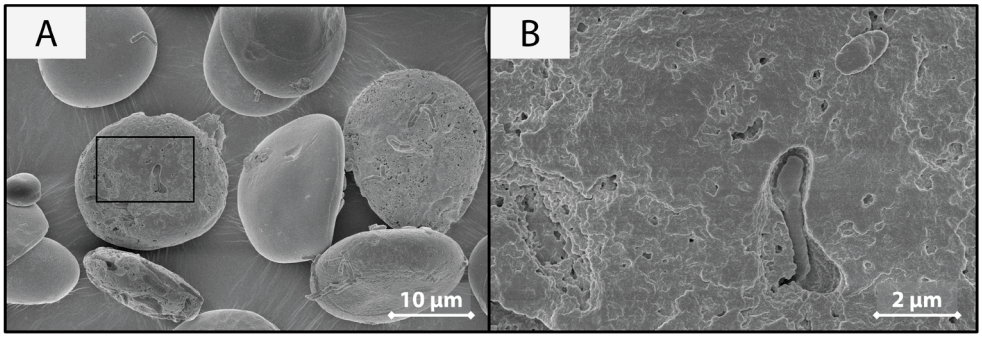

Figure 6.3. SEM images of stomach digesta recovered from pigs fed diets containing isolated barley starch, 5000 times (A) and 25000 times (B) magnified.

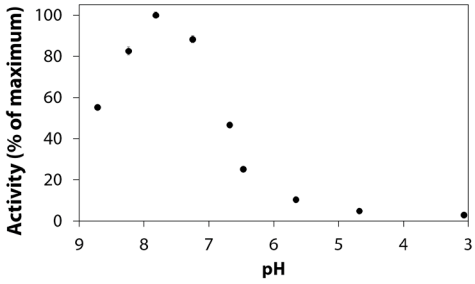

Figure 6.4. Activity of porcine salivary amylase measured at pH 8.5 to 3 expressed as percentage of maximum activity.

glucose and maltose.

Rod-shaped bacterial cells, around 2.5 to 4 µm long, were observed with SEM analysis in stomach digesta of pigs fed ground barley and isolated barley starch. Further SEM analysis revealed cells on the surface of isolated barley starch granules and bacterial cells that were partly embedded in the granules, exposing the layered structure inside the starch granule (**Figure 6.3**). In addition, imprints with a similar shape and size as the rod-shaped bacterial cells were observed on several starch granules. Typically, starch granules that were associated with bacterial cells or imprints of bacterial cells, displayed a more porous granular surface compared with starch granules that were left untouched by bacteria.

Study 2: Salivary amylase characteristics and *in vitro* digestion of starch by enzymes present in saliva and stomach digesta

The DM content of saliva was 12 mg/mL and saliva contained 24.8% protein on a DM basis. The optimum activity of salivary α-amylase on non-reducing-end blocked p-nitrophenyl maltoheptaoside was found to be around pH 7.8 and almost no activity was found below pH 5 (**Figure 6.4**). At pH 7.8, porcine salivary amylase was approximately 20 times more active on pre-gelatinized starch compared to native starch (**Table 6.1**). In contrast, porcine pancreatic α-amylase was around 8 times more active on pre-gelatinized starch compared with native starch, at its optimum pH (pH 7.0). Comparing salivary α-amylase with pancreatic α-amylase activity, we measured a 45 times higher activity for pancreatic α-amylase upon incubation with pre-gelatinized starch and a 108 times higher activity with native starch.

During the 225 min incubation of feed with enzyme extract obtained from stomach digesta, maltodextrins formation was observed mainly for diets with isolated barley starch (up to 11% of starch) and diets with extruded barley (up to 29% of starch, **Figure 6.5**). In contrast, the level of maltodextrins formation after incubation of ground barley did not exceed 2.5%. The formation of maltodextrins from both isolated barley starch and extruded barley increased

Table 6.1. Activity of porcine salivary and pancreatic amylase on pre-gelatinized and native starch at optimum pH, expressed in units (U) per mg DM.[1,2]

Starch	Amylase source	Activity (U/mg DM)	pH of incubation
Pre-gelatinized starch	Porcine saliva	0.90 ± 0.02	7.8
	Porcine pancreas	40.23 ± 1.59	7.0
Native starch	Porcine saliva	0.05 ± 0.07	7.8
	Porcine pancreas	5.37 ± 3.65	7.0

[1] Presented values are averages ± standard deviations.

[2] One unit equals the hydrolysis of one µmole glucose linkages per minute.

rapidly until the pH of the solution was reduced to 5 or lower, where maltodextrin formation levelled off. The starch breakdown products formed upon incubation with stomach digesta extract consisted mainly of maltose, followed by maltotriose and glucose, whereas the recovery of breakdown products with DP>3 was negligible. The concentration of maltodextrins presented are corrected for the amounts formed by acid hydrolysis during blank incubation, which reached cumulative levels up to a maximum of 3% (Supplementary information, **Figure S6.1**).

Upon incubation of feed with porcine saliva, cumulative levels of linear α (1-4) maltodextrins, corrected for acid hydrolysis, reached up to 10% of total starch for extruded barley (**Figure 6.6**). The break down products formed during degradation of extruded barley with saliva consisted mainly of maltose, maltotriose, and maltotetraose, in rather equal amounts. For isolated barley starch and ground barley, cumulative maltodextrins levels amounted to less than 1% of total starch.

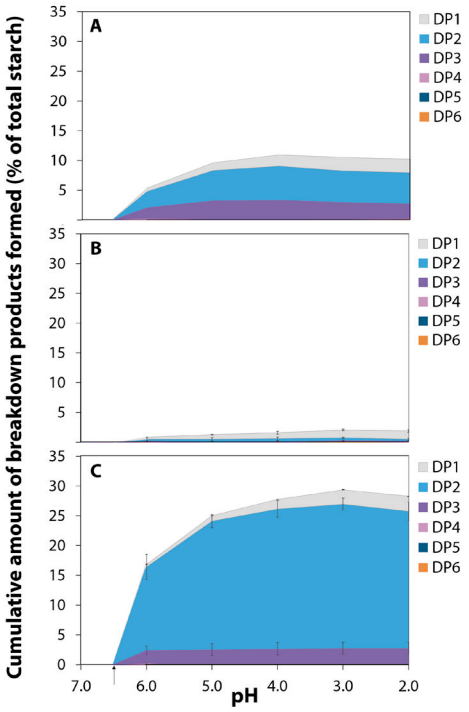

Figure 6.5. Cumulative amount of linear α (1-4) maltodextrins, with a degree of polymerisation (DP) up to 6, formed from starch upon incubation of complete diets containing barley starch included as isolated powder (A), ground cereal (B), or extruded cereal (C). Diets were incubated with an extract obtained from porcine stomach digesta. Starch degradation is visualized during 10 steps of 22.5 min in which the pH is gradually decreased from 6.5 to 2. The arrow indicates the moment of extract addition, the error bars represent the standard error of measured maltodextrins concentrations.

Discussion

The aim of this manuscript is to study the contribution of the stomach on starch disappearance in pigs. To this end, we conducted two studies. In the first study we examined the unabsorbed starch remainders in the stomach of pigs. In the second study we quantified the *in vitro* hydrolysis of starch by enzymes present in saliva and stomach digesta.

In vitro starch hydrolysis by porcine saliva and bacterial enzymes under dynamic stomach conditions

Up to 10% of starch originating from extruded barley was converted by porcine saliva to glucose, maltose, and maltotriose under our dynamic stomach conditions, which is the first report of porcine salivary α-amylase activity under stomach conditions. For human salivary

amylase however, it has been described already that around 50% of starch in bread was converted to oligosaccharides in 90 minutes[11], using a saliva:starch ratio and pH range that were comparable with the ones used in our study. Since the *in vitro* starch digestion rate of bread by porcine pancreatic α-amylase[18] is comparable with that of extruded barley[4], we expected that the extent of starch digestion in our study would be closer to the 50% that was reported earlier. The observed gap of 40% suggests a lower rate of starch hydrolysis by porcine salivary α-amylase compared with human salivary α-amylase. Part of this is explained by the pH optimum of human salivary α-amylase (6.9)[10], which is almost 1 pH unit lower than measured for porcine saliva in this study. Consequently, porcine salivary α-amylase will have a smaller contribution to starch hydrolysis in the stomach than what is currently assumed for human salivary α-amylase.

Up to 29% of starch originating from extruded barley was digested under dynamic stomach conditions by the enzyme extract obtained from stomach digesta. Likely, this extract contains both salivary enzymes and enzymes originating from bacteria, consistent with their presence and activity observed in the SEM photos taken from pig's stomach digesta (**Figure 6.3**). Consequently, both groups of enzymes can contribute to starch digestion by the extract. However, when comparing the breakdown products formed by saliva (**Figure 6.5**) with those formed by the enzyme extract (**Figure 6.6**), a different pattern is observed. This indicates a major contribution of bacterial enzymes to the total starch hydrolysing activity of the enzyme extract. In contrast to saliva, these bacterial enzymes can hydrolyse both gelatinised starch present in diets with extruded barley, as well as native starch present in diets with isolated barley starch. For starch present in ground barley, however, negligible hydrolysis was observed by both bacterial and salivary enzymes. Possibly, the protein matrix, which will not be degraded by porcine pepsin until a pH below 4.0 is reached[19,20], protects starch in ground barley against bacterial and salivary amylase.

Translation of gastric starch hydrolysis measured *in vitro* to the *in vivo* situation

The degree of *in vitro* gastric starch hydrolysis measured in this study, is based on several assumptions made with respect to the pH gradient, incubation time, and concentration of enzymes. Those assumptions were based on our and others earlier observations made about the stomach of pigs and humans. However, the validity of those assumptions will strongly affect the quantitative contribution of salivary and bacterial enzymes to gastric starch digestion *in vivo*.

First, we have likely underestimated the full capacity of gastric bacterial enzymes to hydrolyse starch. The enzyme extract used in the dynamic stomach model was obtained after homogenization of the stomach content. Due to the large variation in anatomy and physiological functions of different regions of the stomach, the amount of bacteria is generally higher in the upper part of the stomach compared with the lower part[12]. Regionally, the bacterial capacity to degrade starch will likely exceed the activity measured in this study. The

ratio between saliva and starch was based on previous studies, which reported that about 0.15[21] to 1 mL[22] of saliva is excreted in pigs per gram feed. Those values are in line with the saliva flow of humans, which ranged from 0.17 to 1 mL per g food[23]. The concentration of saliva used in this study, 1 mL per 1 gram feed, and consequently starch digestion by saliva, might exceed the practical situation in some cases.

Second, the enzyme activity depends on the pH gradient and incubation times used. The total incubation time chosen for the dynamic model used in this study (225 min) is based on the solid digesta mean retention time in the stomach of pigs in our larger *in vivo* trial (187 min on average)[24] and solid digesta retention times reported in other recent studies with pigs (around 200 to 480 min)[25,26]. However, the extent of starch hydrolysis is not limited by the total incubation time, but mainly by the retention time of digesta at pH > 5 (**Figure 6.5**). The stomach pH depends largely on the mixing of the feed bolus, which, in turn, depends on physical digesta properties[27]. Stomach digesta of pigs in this study were rheological characterised as weak gels and had low levels of fluidity[24]. Usually, digesta that behaves as weak gels are not homogeneously mixed in the stomach and pH in the upper part of the stomach will therefore be close to the pH value of the feed[9,27]. In this study, the pH value of the diets ranged from 6.5 to 7.0 and, consequently, a pH of 6.5 was chosen as starting point of each incubation. We assumed a similar gastric pH gradient for each diet in our *in vitro* model. However, the ease of mixing and time to decrease the pH below five may differ between diets. For example, stomach digesta of pigs fed extruded barley, was easier to deform than stomach digesta of pigs fed ground barley and diets with isolated barley starch[24]. Stomach digesta that is easier to deform will have a faster decrease in pH[9] and therefore, our *in vitro* gastric degradation of extruded barley will likely exceed the *in vivo* situation.

In conclusion, gastric bacteria may hydrolyse a considerably fraction of starch, especially

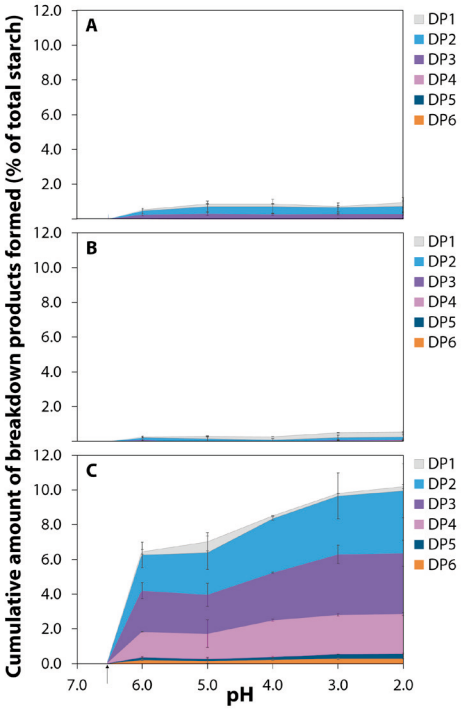

Figure 6.6. Cumulative amount of linear α (1-4) maltodextrins, with a degree of polymerisation (DP) up to 6, formed from starch upon incubation of complete diets containing barley starch included as isolated powder (A), ground cereal (B), or extruded cereal (C). Diets were incubated with porcine saliva. Starch degradation is visualized during 10 steps of 22.5 min in which the pH is gradually decreased from 6.5 to 2. The arrow indicates the moment of saliva addition, the error bars represent the standard error of measured maltodextrins concentrations.

in pigs with stomach digesta that is difficult to mix. Porcine saliva is also able to digest starch under stomach conditions, but to a limited extent, especially in non-processed feed materials. The extent of gastric digestion may vary greatly with the properties of digesta, but gastric bacteria seem easily capable to hydrolyse 10% of native starch fed to pigs into maltodextrins with DP<6. Our current research does not provide further insight in the effect of gastric amylases on starch disappearance in the GIT of pigs, leaving several options open. Breakdown products of starch may be directly utilized by bacteria or they may be hydrolysed further by endogenous enzymes of the pig. Glucose utilization by gastric bacteria could explain differences found between glucose recovery measured in the portal vein and ileal starch digestibility: Across several studies[28-32], between 60 to 100% of ileal digestible starch is recovered in the portal vein. The splanchnic metabolism is partly responsible for this gap, but the total amount of glucose used for the splanchnic metabolism is estimated to account for 17% of ingested starch[31], and thus does not fully cover this gap in all cases. In addition to utilization of starch by bacteria, gastric bacteria may have a synergistic effect on starch hydrolysis kinetics in pigs. Partly hydrolysed starch granules may be more rapidly digestible by porcine saliva or pancreatic amylases. This would partly explain the unexpected high levels of starch hydrolysis observed previously in the proximal small intestine of pigs[1-4].

Conclusions

In this study, we found that porcine salivary α-amylase is most active at a pH of 7.8 and that it can, to a limited extent, hydrolyse processed starch under stomach conditions. Bacterial enzymes, extracted from stomach digesta of pigs, were able to digest both native starch and processed starch in our dynamic stomach model. Both porcine saliva and bacterial enzymes mainly hydrolysed starch above pH 5 and, consequently, the contribution of gastric digestion to the total *in vivo* starch disappearance will depend greatly on the pH gradient in the stomach. Assuming that the pH of gastric digesta remains above pH 5 for at least one hour, we found that up to 30% of starch may be degraded when pigs are fed a processed starch. This implies that the stomach has a considerable role in starch digestion kinetics in pigs, which is currently underestimated in most commonly used *in vitro* methods.

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SUPPLEMENTARY INFORMATION

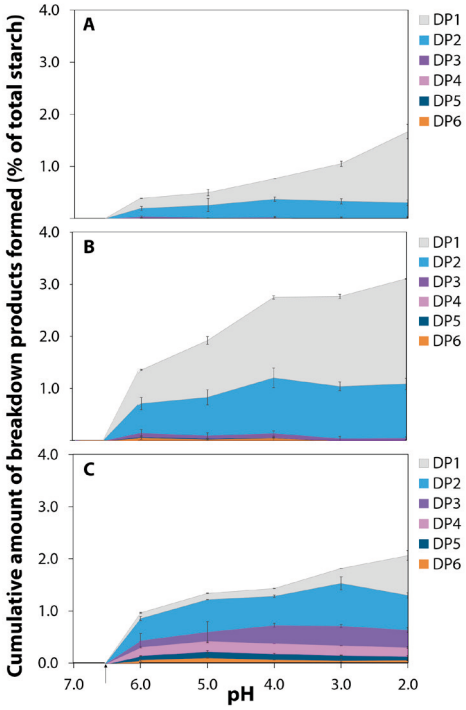
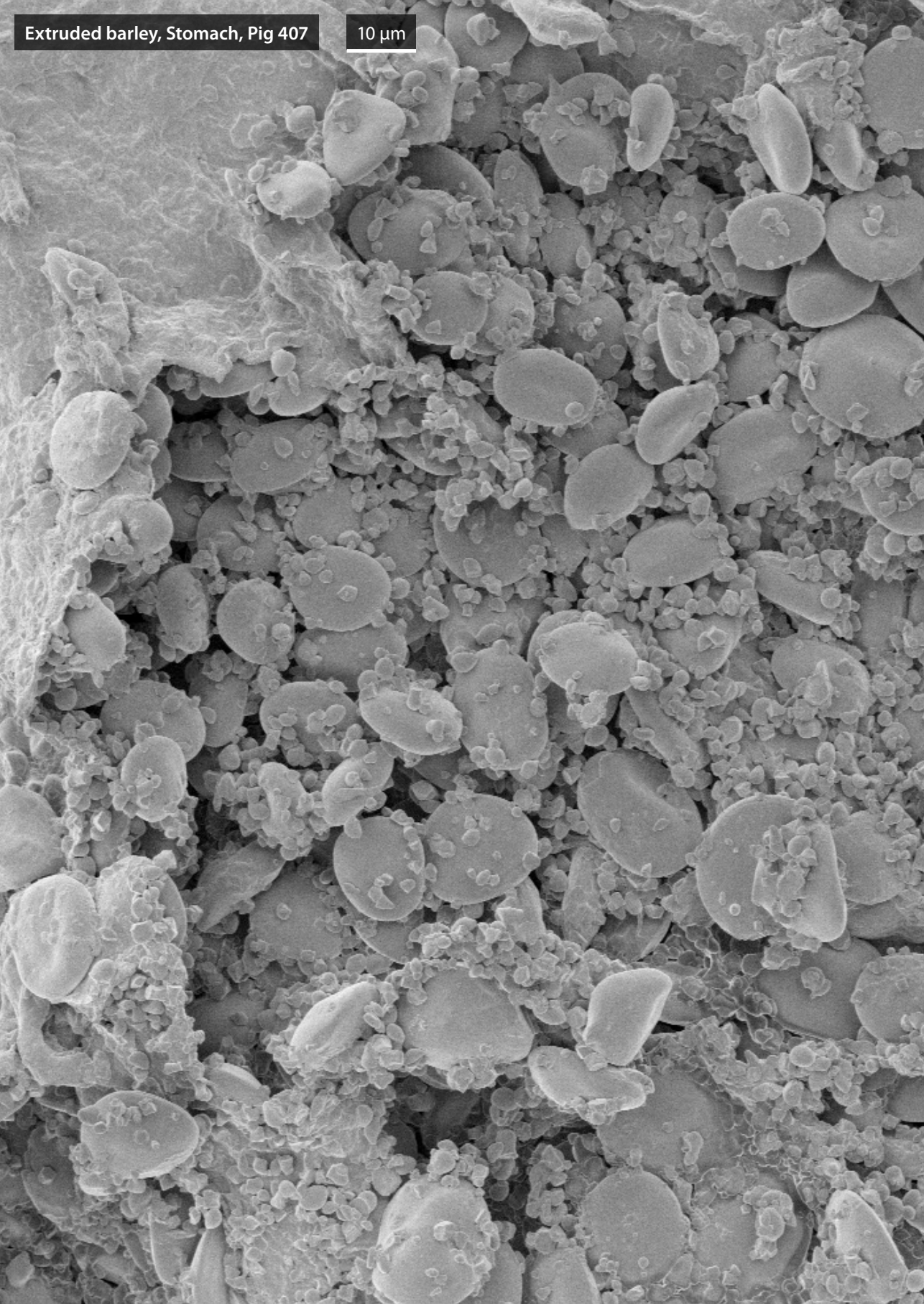


Figure S6.1. Cumulative amount of linear α (1-4) maltodextrins formed from starch in complete diets containing barley starch included as isolated powder (A), ground cereal (B), or extruded cereal (C). No amylolytic enzymes were added to the incubation. Starch degradation is visualized during 10 steps of 22.5 min in which the pH is gradually decreased from 6.5 to 2. The error bars represent the standard error of measured maltodextrins concentrations.


Extruded barley, Stomach, Pig 407
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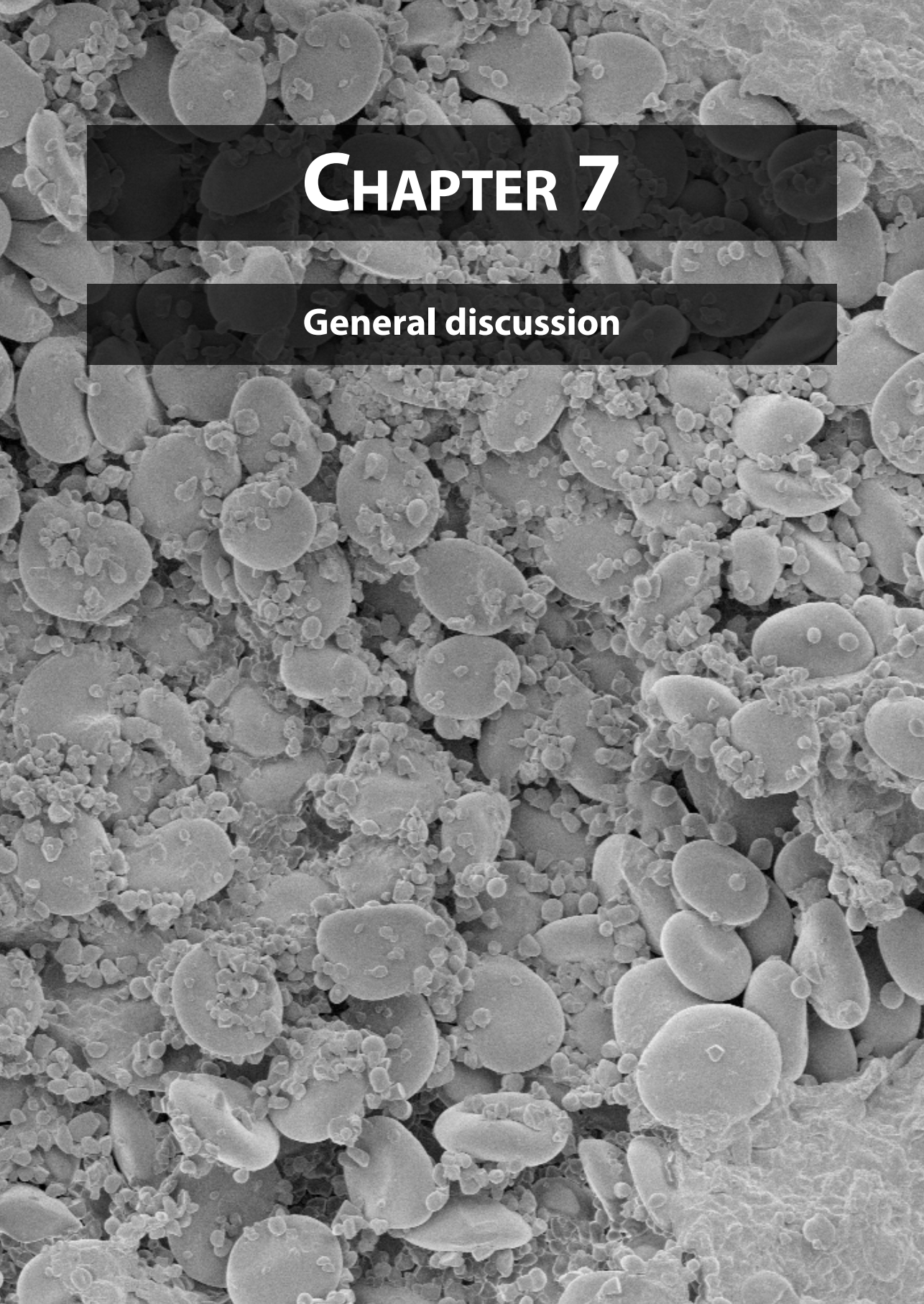

Chapter 7

General discussion

AIM AND MAIN CONCLUSIONS

The main aim of this thesis was to quantify the contribution of intrinsic starch structure, feed processing, and digesta passage behaviour on the kinetics and mechanisms of starch digestion in the upper gastrointestinal tract (GIT) of pigs. Research in **Chapter 2** revealed that structural properties of starch that affected *in vitro* hydrolysis kinetics, depend greatly on the botanic source that the starch originates from. Across botanic sources, the hydrolysis rate increased by increasing concentrations of A-type crystalline structure and short amylopectin side-chains. Within maize starches, however, an increased *in vitro* hydrolysis rate of starch was additionally explained by a decreased amylose content and an increased number of pores. Consisted with these *in vitro* findings, an increased amylose content negatively affected the digestion rate of maize starches in pigs, as described in **Chapter 3**. Extrusion increased digestibility in proximal and distal parts of the small intestine, whereas the cereal matrix of maize resulted in 16% starch that was resistant to digestion, which was not observed for barley. In addition, a substantial amount of starch (up to 25% of ingested starch) was present as soluble maltodextrins in the proximal small intestine. In **Chapter 4**, we concluded that digesta mean retention times (MRT) of pigs were longer for the stomach (129 to 225 min for solids) than for the small intestine (86 to 124 min for solids). In addition, liquids remained around one hour shorter in the stomach than solids. Solid digesta MRT in the stomach was positively correlated with the shear stress required to deform digesta. However, against expectation, a stronger relation was observed between digesta MRT and the saturation ratio (SR), which is the actual amount of water in stomach digesta as a fraction of the theoretical maximum held by digesta dry matter. In contrast to pigs fed extruded cereals, a high digesta SR and shear stress were observed for pigs fed diets with isolated starch or ground cereals. The ^{13}C breath test, described in **Chapter 5**, revealed a biphasic pattern of gastric emptying after meal feeding and we estimated that around 60% of the meal was emptying during the first phase. The first peak in gastric emptying of solids reached its maximum within two hours after feeding in all cases. For digesta liquids, this peak was reached 0.6 hours earlier for pigs fed ground barley, compared with pigs fed diets containing isolated starch. Diets ranked similar in gastric MRT after continuous feeding as they did in time to reach the second peak after meal feeding, but not in the time to reach the first peak after meal feeding. Small intestine digesta retention times presented in **Chapter 4** enabled the calculation of starch hydrolysis kinetics, which is presented in **Chapter 3**. Starch hydrolysis in the proximal small intestine was underestimated by standard *in vitro* approaches, whereas the amount of starch resistant to hydrolysis by endogenous enzymes exceeded our *in vitro* predictions. In addition, glucose release from slowly digestible starch is less gradual than predicted from *in vitro* analysis. Based on the large underestimation of starch disappearance in the proximal small intestine, we hypothesized that the role of the stomach on *in vivo* starch disappearances might currently be underestimated, which is elaborated in **Chapter 6**. We found evidence of granular starch degradation by bacteria in the stomach of pigs using electron microscopy. Bacterial enzymes, extracted from stomach digesta of pigs, hydrolysed both native and processed starches (up

to 29%) when the pH was step-wise decreased from 6.5 to 2.0 in 225 min. Porcine salivary α-amylase, which has an optimum pH around 7.8, degraded up to 10% of gelatinized starch under stomach conditions, whereas its effect on native starch seems negligible.
At this moment, the understanding of starch digestion kinetics is hampered by the limited knowledge of starch hydrolysis and digesta behaviour in the stomach and proximal small intestine. Hence, in this final chapter I will revise how the results of this thesis contribute to a better understanding of the unexpected rapid digestion of starch in the proximal GIT.

Starch digestion (re)defined

The term starch digestion is frequently used in various studies, including most chapters of this thesis. The meaning of this term, however, is ambiguous across studies. This ambiguity originates from the multiple disciplines that target starch digestion and from the many different methods that can be used to quantify digested starch. From the physiological perspective, "digested" starch is starch that has disappeared from the GIT as a result of hydrolysis by endogenous enzymes into glucose. From the enzymatic perspective, "digested" starch is intact starch that has been hydrolysed into soluble break down products such as glucose and maltodextrins. In scope of the multidisciplinary origin of this PhD thesis, several definitions and methods are discussed here, to accommodate an unambiguous description of starch digestion in pigs.

Definitions to describe starch digestion in pigs

1. **Starch digestion**: The *in vivo* disappearance of glucose moieties of starch from the small intestine as a result of enzymatic hydrolysis by endogenous enzymes of the pigs.
2. **Starch disappearance**: The *in vivo* disappearance of starch from the GIT by either digestion or bacterial fermentation.
3. **Enzymatic starch hydrolysis**: The degradation of starch into intermediate products, limit dextrins, and glucose, by endogenous enzymes in the pig or in *in vitro* models.
4. **Net glucose appearance**: the *in vivo* appearance of glucose in the portal circulation.

Measurement techniques to quantify digested starch

The main challenge associated with the fractions defined above, is the quantification of each fraction, which depends on the choice and availability of measurement techniques. Most complicated to quantify is the fraction of digested starch as defined above, which can not be measured by a single method. By combining measurements of disappeared starch, enzymatically hydrolysed starch, and glucose that appeared in the portal vein, one can obtain a much more accurate insight in the digestion of starch by pigs (**Figure 7.1**).
Disappeared starch is usually calculated as 100% minus the starch that is still present in digesta

harvested from a particular location inside the GIT. Depending on the method of analysis, the amount of starch that is defined as disappeared starch can either include or exclude glucose and soluble glucose oligomers. If digesta is washed with ethanol prior to starch analysis (ZETam in the Dutch feed evaluation system[1]), the amount of starch quantified as remaining starch excludes glucose and soluble oligomers and thus the amount of starch quantified as disappeared starch includes glucose and soluble oligomers. If digesta is not washed prior to starch analysis (ZETtot in the Dutch feed evaluation system[1]), the amount of disappeared starch excludes glucose and oligomers. Note that the abbreviation DC, used to denote a Digestibility Coefficient, could effectively be used to denote a "Disappearance Coefficient". Only under the assumption that disappearance of starch results from hydrolysis by endogenous enzymes of the pig, starch disappearance, excluding glucose and oligomers, equals starch digestion. Throughout chapter 3 we did not challenge this assumption because we did not have enough information on the role of starch fermentation in the upper GIT. However, as shown in chapter 6, gastric bacteria can utilize starch in the upper GIT, which leads to an overestimation of the quantity of truly digested starch when measuring only the fraction of disappeared starch in the small intestine of the pig.

Glucose appearance in the portal circulation is amongst the closest things that can be measured *in vivo* to quantify the true amount and rate of starch digestion. Portal glucose levels can be used to distinguish disappeared starch from digested starch, as the amount of starch that is consumed by bacteria will not show up as glucose in the portal circulation. Glucose appearance, however, does not directly reflect starch digestion, as it excludes the consumption of glucose by intestinal tissues (either from absorbed or arterially supplied glucose)[2] and includes the possible production of glucose from other nutrients in intestinal tissues.

In addition to its *in vivo* application, the term digested starch is used in many *in vitro* models. According to the proposed definitions, the amount of digested starch in *in vitro* models should be described as hydrolysed starch. In the *in vitro* small intestine model used in this thesis, an overdose of amyloglucosidase rapidly converts end products of pancreatic α-amylase hydrolysis into glucose. Therefore, the amount of hydrolysed starch in the *in vitro* small intestine model can be measured by the total amount of glucose that is released. In our *in vitro* stomach model, a rather unknown cocktail of enzymes was extracted from stomach contents. In that case, starch hydrolysis refers to the end products that are typically formed by amylolytic enzymes: glucose and glucose oligosaccharides with DP<6. In the pigs small intestine, a dominant role of α-amylase and brush border enzymes on the total enzymatic hydrolysis is assumed. Hydrolysed starch as measured in the small intestine equals end products of these enzymes, which are mainly glucose and limit dextrins.

To compare *in vitro* models with the *in vivo* situation, it is important that similar starch fractions are considered. As shown in chapter 3, the differences between the amount of starch that is hydrolysed and the amount that is disappeared can be as much as 25% of the starch included in the diet, depending on the location inside the gastrointestinal tract. For this thesis, glucose produced *in vitro* should be compared with the amount of starch, including glucose and limit dextrins, that disappeared from the small intestine of pigs.

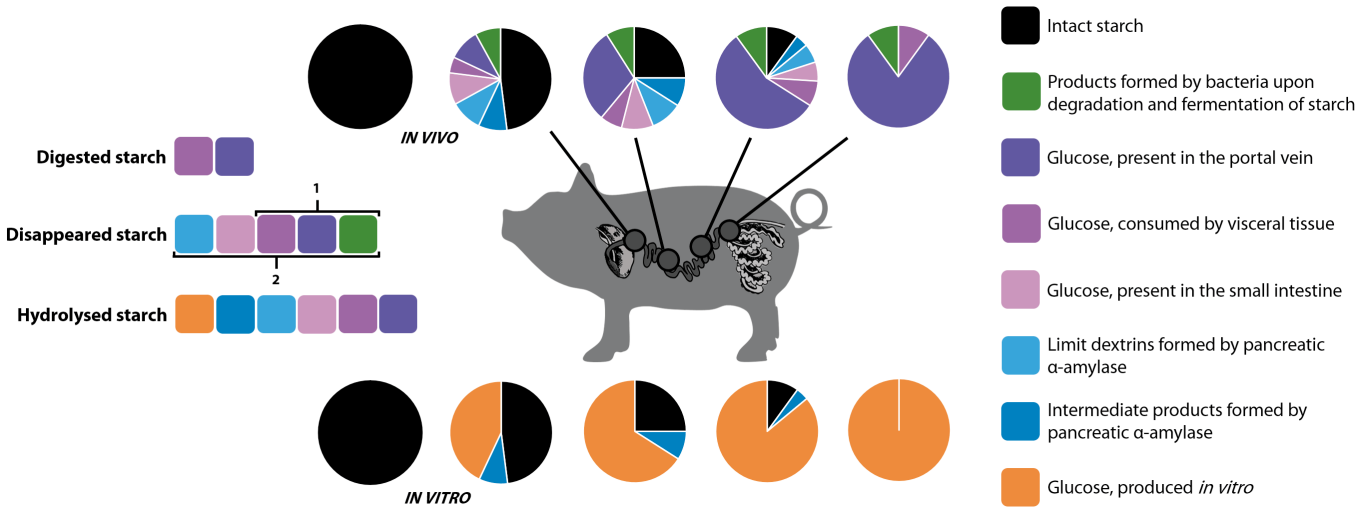

Figure 7.1. Proposed definitions for starch digestion, starch disappearance, and starch hydrolysis. The circle diagrams represent a theoretical mass balance of intact starch and products formed from starch during digestion. These include products that are formed by endogenous enzymes, such as glucose and glucose oligomers or polymers, or products that are formed by starch degradation and fermentation by bacteria, such as bacterial biomass or volatile fatty acids. *In vivo* stands for the small intestine of a growing pig, whereas *in vitro* refers to the small intestinal model used in this thesis, in which starch is digested by a combination of pancreatic α-amylase and amyloglucosidase.
[1]Disappeared starch as calculated when glucose and soluble oligomers are not removed from digesta that is recovered from the GIT (100%-ZETtot according to the Dutch feed evaluation systems).
[2]Disappeared starch as calculated when glucose and soluble oligomers are removed from digesta that is recovered from the GIT (100%-ZETam according to the Dutch feed evaluation systems).

Table 7.1. Digestion coefficients (DC) of starch and concentrations of starch, chrome and cobalt in feed and in digesta recovered from the stomach of pigs fed diets containing barley, maize, or high amylose maize starch, included as isolated powder, ground cereal, or extruded cereal.[1,2]

	Experimental treatment[3]									S	P – value[4]		
	Barley			Maize			High amylose maize						
	I	G	E	I	G	E	I	G	E		Form	Source	F*S
Stomach digesta													
Observations	10	10	9	10	10	9	7	7	10				
DC starch	0.23^{a}	-0.28^{b}	-0.49^{bc}	0.20^{a}	-0.46^{bc}	-0.46^{bc}	0.05^{a}	-0.75^{d}	-0.65^{cd}	0.15	**<0.0001**	**0.0001**	**0.030**
Starch (g/kg DM)	267^{d}	393^{c}	469^{b}	293^{d}	486^{ab}	476^{ab}	292^{d}	503^{a}	504^{a}	20	**<0.0001**	**<0.0001**	**<0.0001**
Of which soluble[5] (%)	2	2	12	3	1	17	3	2	6				
Chrome (mg/kg DM)	$159^{kl|xy}$	$134^{l|xy}$	$122^{l|xy}$	$166^{kl|x}$	$138^{l|x}$	$127^{l|x}$	$148^{kl|y}$	$122^{l|y}$	$123^{l|y}$	16	**<0.0001**	**0.008**	0.570
Cobalt (mg/kg DM)	131^{ab}	85^{d}	128^{ab}	141^{a}	96^{cd}	117^{bc}	131^{ab}	90^{d}	92^{d}	14	**<0.0001**	**0.009**	**0.002**
Diets													
Starch (g/kg DM)	423	444	470	423	472	482	401	467	474				
Chrome (mg/kg DM)	189	190	181	190	191	182	190	192	182				
Cobalt (mg/kg DM)	189	190	181	190	191	182	190	192	182				

[1] Presented values are estimated means and standard deviations (S).

[2] DC values are calculated using the dual-marker method[1].

[3] Starch forms are abbreviated as follows: isolated (I), ground (G), and extruded (E) form.

[4] p-values for fixed effects of starch form (isolated, ground vs extruded), source (barley, corn vs. high amylose corn), and the interaction between form and source, analysed per segment. When an interaction between form and source was found, superscripts ^{a,b,c,d} indicate significant differences between treatment combinations ($P<0.05$). In the absence of source*form interactions, superscripts ^{k,l,} are used to indicate significant differences between starch forms ($P<0.05$) and superscripts ^{x,y,z} indicate significant differences between starch sources ($P<0.05$).

[5] The percentage of starch present as glucose and soluble maltodextrins is determined in stomach digesta pooled per treatment and corrected for the amount of glucose and maltodextrins present in the diets.

Starch digestion coefficients: methodology

The golden standard to measure starch DC today is the quantitative or total collection method[3]. This method requires accurate recording of the total feed intake and faecal output during a certain period. Nutrient concentrations in both feed and faeces are measured and multiplied with the quantity of feed consumed and faeces produced, respectively, which is laborious and requires specially designed animal trial facilities. As a result, the total collection method has lost terrain to the marker method. The marker method is based on the inclusion of an indigestible compound (marker or tracer) in the diet, which can be used to calculate the disappearance of a nutrient by expressing the concentration of a nutrient in both the diet and faeces relative to the concentration of indigestible marker[3].

The marker method offers several benefits. For example, it enables the calculation of DC in intermediate parts of the GIT. Subsequently, the marker technique became a popular method to determine ileal digestibility of nutrients[4]. A well-known challenge of the marker method, is the assumption that the marker and nutrient of interest travel through the GIT at the same speed. Due to the high level of digesta homogeneity and the straightforward, tubular flow in the lower GIT, this seems a safe assumption for faeces and ileal digesta. Indeed, the marker technique, compared with the total collection method, has resulted in similar DC values for ileal digesta[4] and faeces[3-5]. However, the assumption that marker and nutrient behave similar is not always true early in the upper GIT[6], which possibly affects starch DC in the proximal small intestine and stomach. To overcome part of the differences in behaviour between the marker and starch, the dual marker technique was used in this thesis. This technique corrects for a higher passage rate, and thus decreased representation, of nutrients that are solubilized during digestion[6].

When assuming disappearance of starch in the stomach due to fermentation by gastric bacteria (chapter 6), starch DC values are expected to be somewhat positive. The measured starch DC in the stomach of pigs, however, varied to extents that are physiological unrealistic (**Table 7.1**). Especially the starch DC in the stomach of pigs fed ground and extruded cereals, which varied from -0.28 to -0.75, seems erroneous. Physical properties, such as particle size, affect gastric retention times of insoluble particles[7]. Consequently, the concentration of starch in the stomach of pigs fed ground and extruded cereals decreased, relative to concentrations in the diet, less rapidly than chrome and cobalt concentrations. This accumulation of starch has led to negative DC values. In contrast, positive DC values were observed for pigs fed diets with isolated starches. In those pigs, both starch and markers are much smaller than 1 mm and therefore expected to leave the stomach with approximately the same flow rate. This suggest that a much larger part of the starch than expected, up to 23%, truly disappears in the stomach of pigs.

It is unsure how differences in passage behaviour of tracer and tracee affect the calculations of starch DC in the proximal small intestine[6,8-10]. In a steady state, small intestine digesta is much more homogeneous than stomach digesta, because of the sieving effect of the stomach. Consequently, flow rates of starch and markers in the small intestine, and thus the calculation of DC values, are assumed to be unaffected by physical digesta properties in this study.

Text box 7.1

ESTIMATION OF *IN VIVO* STARCH HYDROLYSIS KINETICS

To estimate the *in vivo* hydrolysis rate of starch, the hydrolysis coefficient and cumulative retention time of starch in the small intestine of growing pigs were calculated according to the following equations:

Equation 7.1. $$HC(n) = 1 - \left(\frac{[Cr_F] * (1 - S)[starch_D]}{[Cr_D] * [starch_F]} + \frac{[Co_F] * (S)[starch_D]}{[Co_D] * [starch_F]} \right)$$

Where HC(*n*) is the hydrolysis coefficient of starch in the compartment *n*, which is the amount of starch that is disappeared or hydrolysed into maltodextrins with DP≤3, as fraction of starch ingested. [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 intact starch and starch break down products with a degree of polymerisation (DP) ≥ 3 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.

Equation 7.2. $$CRT_H(n) = S * \left(MRTl(n-1) + 0.5 * MRTl(n)\right) + (1 - S) * (MRTs(n-1) + 0.5 * MRTs(n))$$

Where CRT_H is the cumulative retention time of digesta in SI compartment *n* in minutes, and S is the fraction of soluble starch breakdown products with DP≥3 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. For SI1, MRT(n-1) is zero.
All data points obtained from **Equation 7.1** and **Equation 7.2** per dietary treatment were used to fit a first order kinetics model:

Equation 7.3. $$HC = HC_{intital} + plateau_H * (1 - \exp(-\frac{K_H}{Plateau_H} * CRT_H))$$

Where HC is the hydrolysis coefficient of starch calculated with **Equation 7.1**. $HC_{initital}$ is the estimated amount of starch that is disappeared or hydrolysed into maltodextrins with DP≤3 at the moment digesta entered the small intestine. Plateau is the maximum amount of starch hydrolysed as % of starch ingested. K is the rate of starch hydrolysis or disappearance, corrected for plateau effects, as % of total starch hydrolysis in glucose per minute. CRT is the cumulative retention time of digesta (in minutes) as calculated with **Equation 7.2**. All parameters were estimated by nonlinear regression procedures (PROC NLIN, SAS, version 9.4, SAS Institute, Cary, USA).
Two examples of the fit of **Equation 7.3** and the subsequent estimation of the amount of starch hydrolysed in 20 minutes (RHS) is shown in **Figure 7.2**.

STARCH HYDROLYSIS KINETICS

The definitions rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) are widely used to describe *in vitro* determined starch digestion rates[11]. In line with the newly proposed definitions, these fractions will be referred to as rapidly hydrolysed starch (RHS), slowly hydrolysed starch (SHS), and starch resistant to hydrolysis (RS_H), from this point onwards. *In vitro*, RHS is the amount of starch that is hydrolysed within 20 minutes by pancreatin α-amylase to yield mainly maltose and higher maltodextrins. SHS is the amount of starch hydrolysed between 20 and 120 minutes, whereas RS_H is the amount of starch that is not hydrolysed within 120 minutes.

A growing number of studies focuses on translation of those *in vitro* classified fractions of starch to the *in vivo* rates of starch digestion. These studies, however, do not provide full insight in *in vivo* rates of starch hydrolysis as information on the concentration of starch break down products and/or small intestine digesta retention time is missing[12-14]. In this thesis I presented *in vivo* hydrolysis data and small intestine digesta retention times in pigs (chapter 3), which allowed for the calculation of an *in vivo* hydrolysis rate and *in vivo* levels of RHS, SHS, and RS_H (**Text box 7.1** and **Figure 7.3**). For *in vivo* starch hydrolysis, I defined RHS as the amount of starch that is hydrolysed into end products of pancreatic α-amylase within 20 min after digesta enters the small intestine. RS_H refers to the amount of starch that is not hydrolysed at the ileum site of the small intestine of pigs. Consequently, *in vivo* SHS is the difference between RHS and the fraction of starch that is hydrolysed at the ileum. For all non-hydrothermally treated starches, RHS is underestimated *in vitro*, whereas RS_H is usually overestimated, which typically leads to an overestimation of SHS.

The overall *in vivo* rate of starch digestion is higher than the *in vitro* rate of starch digestion for all non-hydrothermal treated starches (**Table 7.2**). In addition, a major part of starch is estimated to be disappeared or hydrolysed at the moment digesta enters the small intestine ($HC_{initial}$). This fraction includes starch that is utilized and hydrolysed by gastric bacteria and salivary amylase, but the extent of gastric hydrolysis (**Table 7.1** and Chapter 6) does not seem

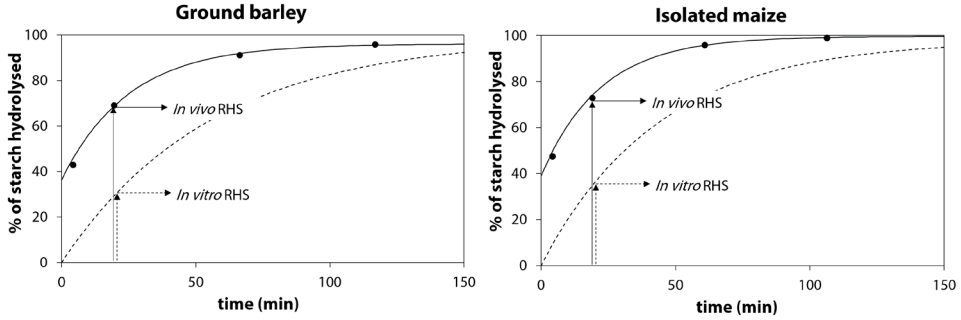

Figure 7.2. *In vivo* (solid line) and in *vitro* (dashed line) modelled starch hydrolysis kinetics and rapidly hydrolysed starch (RHS). The dots represent the LS means of *in vivo* determined DH in four parts of the small intestine. *In vivo* starch hydrolysis is plotted against the CRT, whereas *in vitro* starch hydrolysis is plotted against incubation time.

Table 7.2. Estimated hydrolysis coefficient of starch at the moment digesta enters the small intestine ($HC_{initial}$) and overall *in vivo* and *in vitro* rates of starch hydrolysis.

Experimental diet	***In vivo*** $HC_{initial}$	***In vivo*** rate (%/min)	***In vitro*** rate (%/min)
Isolated barley	0.54	6.5	4.0
Ground barley	0.36	4.1	1.8
Extruded barley	0.61	4.4	15.1
Isolated maize	0.39	4.3	2.3
Ground maize	0.39	6.7	1.8
Extruded maize	0.66	6.4	13.0
Isolated high amylose maize	0.25	2.3	0.4
Ground high amylose maize	0.21	7.0	0.4
Extruded high amylose maize	0.66	3.4	7.1

to cover the total amount of $HC_{initial}$. The model used to fit *in vivo* hydrolysis data will greatly affect $HC_{initial}$. A first order kinetics model was used because of its widely accepted fit for *in vitro* hydrolysis kinetics[15,16]. Below, *in vivo* phenomena are discussed that challenge the suitability of a first order kinetics model to describe *in vivo* starch hydrolysis, such as gastric pre-digestion of starch and the synergistic action of α-amylase and brush-border enzymes. Later on, potential causes for the *in vitro* overestimation of SHS and RS will be discussed.

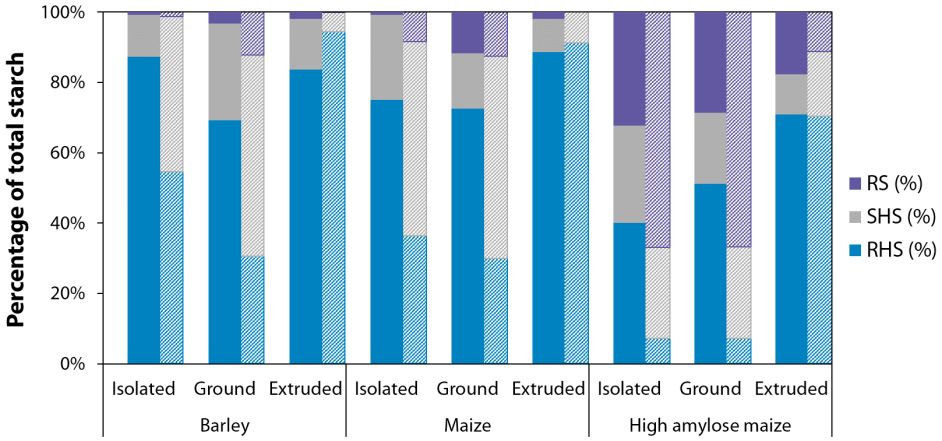

Figure 7.3. *In vivo* (solid) and *in vitro* (striped) rapidly hydrolysed starch (RHS), slowly hydrolysed starch (SHS), and starch resistant to hydrolysis (RSH) as percentage of total starch in diets containing barley, maize, or high amylose maize starch, included as isolated powder, ground cereal, or extruded cereal. Values are calculated according to the methods described in Chapter 3 (*in vitro*) or **Textbox 7.1** (*in vivo*).

Overestimation of rapidly hydrolysed starch

Gastric pre-digestion of starch

The presence of surface pores is considered to be an important factor in the hydrolysis rate of maize starch (Chapter 2 and other studies[17,18]). The presence of surface pores regained attention after Fannon's publication in 1992[18], after which evidence on the presence of interior channels and cavities, openings in starch granules beyond the surface of granules, followed soon[19-21]. Most of the techniques used to study channels and cavities include some sort of (fluorescent) dye that colours the internal surface of starch granules. Such a dye only reaches cavities that are in contact with the surface via channels. Consequently, knowledge on internal channels or cavities that do not reach the surface is scarce. In our research, analysis of gastric digesta of pigs fed high amylose maize (**Figure 7.4**) and barley (data not shown) suggests that gastric bacteria create or uncover pores by removing the surface layer of the starch granule. These phenomena would explain our observations in the proximal small intestine of pigs, where part of the high amylose starch granules (**Figure 7.4**) and barley starch granules (data not shown) seemed richer in surface pores compared with granules in the diet. A similar change in granular surface was reported recently for barley starch in the rumen of heifers[22]. This suggests that the interior of starch granules is loosely packed an consists of an network of internal channels that do not reach the surface of the starch granule. If pores are indeed a

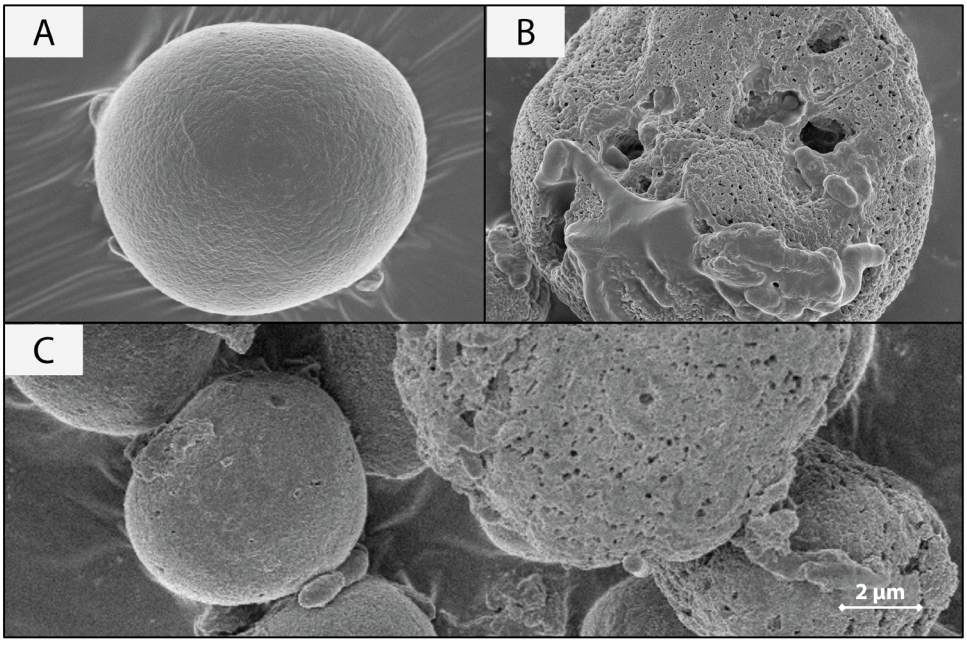

Figure 7.4. High amylose maize starch granules in the feed (A) or recovered from digesta in the stomach (B) or proximal small intestine (C).

prerequisite for (rapid) hydrolysis, this *in vivo* increase in porosity, caused by gastric bacteria, could explain part of the differences between *in vivo* and *in vitro* levels of RHS observed for HA maize and barley starch (**Figure 7.3)**.

Brush border enzyme activities in the small intestine

Previous *in vitro* studies have shown that a combination of pancreatic α-amylase and brush border enzymes can digest starch synergistically and that brush border enzymes can even hydrolyse native starch granules[23]. For example, unique granular surface patterns were visualized on native starch granules that were caused by hydrolysis by human small intestinal maltase-glucoamylase[24]. These patterns show a remarkable similarity to the surface patterns of some granules observed in the proximal small intestine of pigs in our trial (**Figure 7.5**). This unique surface pattern was observed only for pigs fed high amylose starches, and not for pigs fed any of the other starch sources. This suggests that the contribution of brush border enzymes on total starch hydrolysis depends on the intrinsic properties of starch.
The activity of brush border enzymes on the native starch granule is much lower than the activity of porcine α-amylase[25]. This implies a limited contribution of direct granular hydrolysis by brush border enzymes to overall starch hydrolysis in the proximal small intestine. However, a recent study revealed that the amount of branches and the distance between branches in hydrolysis products of α-amylase determines the glucose release rate of maltase-glucoamylase[26]. This suggests that the overall rate of starch hydrolysis does not depend solemnly on α-amylase, but determined by the combination of α-amylase and brush border enzymes. Indeed, others have found a different ranking in starch hydrolysis rate for starches incubated with porcine pancreatic α-amylase than for starches incubated with both pancreatic α-amylase and brush border enzymes from rat intestine[25]. In light of the differences in *in vitro* and *in vivo* RHS, especially this last discovery is alarming. The level of RHS of pure barley and maize starches were, in this thesis, underestimated by 33-39 percentage points. As indicated above, it seems that solemnly gastric (pre)digestion of starch cannot explain this difference.

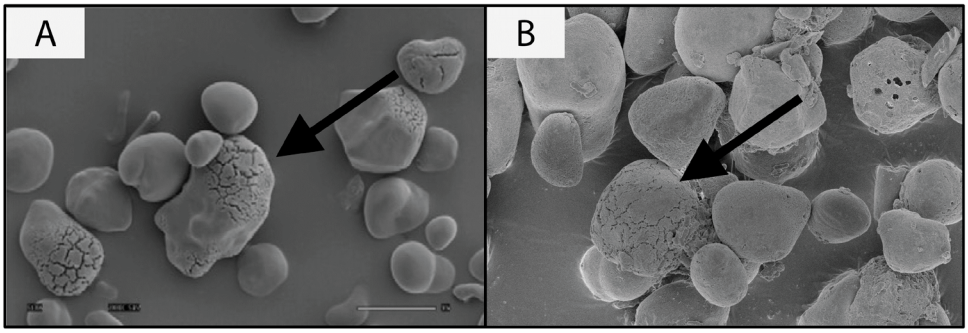

Figure 7.5. Panel A: Surface hydrolysis of high amylose maize starch by a recombinant N-terminal subunit enzyme of human small intestinal maltase-glucoamylase, reproduced with permission[24]. Panel B: Starch granules recovered from the proximal small intestine of a pig fed high amylose maize (this thesis). The granule indicated with the arrow has a similar surface pattern as the granule in panel A.

Therefore, the underestimation of RHS with our *in vitro* assay may be additionally related to the absence of brush border enzymes, which mode of action seems to depend greatly on the molecular and granular structure of starch.

Slowly hydrolysed starch and starch resistant to hydrolysis

In vivo RS_H concentrations were overestimated for pigs fed ground barley, ground high amylose maize, or diets with isolated maize starch or isolated high amylose maize starch by our *in vitro* digestion model (**Figure 7.3)**. In contrast, the *in vivo* RS_H concentrations were slightly underestimated for pigs fed extruded high amylose maize. Arguably, there are slight differences in incubation times of the *in vitro* assay and digesta retention times of the *in vivo* situation (Chapter 4). To this end, we calculated the amount of starch hydrolysed *in vitro* at an incubation time that equalled the total MRT of digesta in the small intestine. This, however, did not notably improve the predictability of our *in vitro* assay. Two factors that possible affect SHS and RS_H predictability are discussed below: The granular structure, especially that of high amylose starch, and the endosperm structure of the cereal kernel.

Effect of granular structure

The amount of isolated high amylose starch that was disappeared cumulatively at the third part of the small intestine (58%) was remarkable close to the total amount of starch that was disappeared at the ileum (60%) (Chapter 3). Seemingly, in the third part of the small intestine, almost all non-resistant starch has disappeared, leaving around 40% starch that is truly resistant to endogenous porcine enzymes. Microscopic analysis of undigested starch residues revealed that most high amylose maize starch granules in the third part of the small intestine, and all granules in the last part of the small intestine, were fully intact and had no pores at all (Chapter 3). Possible, those non-porous intact granules were simply left untouched by gastric bacteria, leaving them resistant to pancreatic α-amylase digestion. Alternatively, certain granules of high amylose starch may have an other structural property than others, which leaves them resistant to hydrolysis by α-amylase. Further microscopic analysis revealed that partly hydrolysed high amylose maize starch granules in the proximal small intestine (**Figure 7.6**, panel B), have a highly similar digestion pattern as observed for normal maize starch granules (**Figure 7.6**,

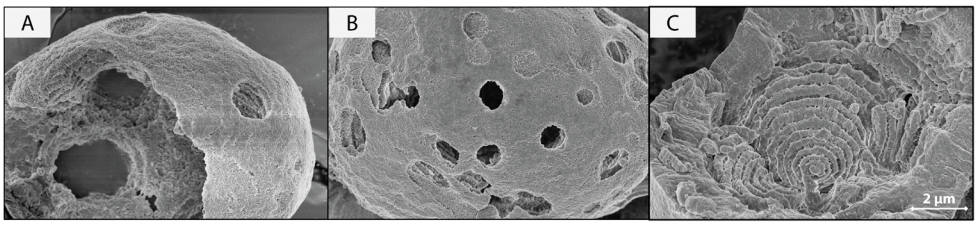

Figure 7.6. Panel A: partly digested granule originating from maize starch. Panel B: partly digested granule originating from high amylose maize starch. Panel C: remainder of a starch granule originating from high amylose maize starch. All starch fragments are recovered from small intestinal digesta of pigs studied for this thesis.

panel A) and barley starch granules (not shown): These granules display several circular erosion areas, which penetrate trough one or more layers and subsequently produce a hollow granule, as reported previously for barley starch[27]. In contrast, some granules of high amylose maize starch contained a crystalline layer on the outer part of the granule that is much thicker than the other layers observed in maize and high amylose maize granules (**Figure 7.6**, panel C). Contrasting to the resistant high amylose starch granule in panel C of **Figure 7.6**, both granules in panel A and B have an homogeneous layer distribution within each granule. An heterogeneous layer thickness within one granule was identified previously in granules of several types of α-amylase resistant high amylose maize starches[28], but the exact structural differences between the different layers remains unknown. Possible, this outer layer prevents pancreatic α-amylase in its conventional method of granular digestion and protects the remaining semi-crystalline structure inside the granule from α-amylase hydrolysis.

Effect of endosperm structure on variation in starch digestion kinetics

The *in vitro* starch hydrolysis rate of ground barley was highly similar to that of ground maize. However, *in vivo,* ground barley was hydrolysed to a larger extent than ground maize. The *in vitro* breakdown of the cereal matrix does not seem to correspond well with the *in vivo* degradation. In our *in vitro* method, the cereal matrix is solemnly degraded physically, due to the inclusion of glass marbles. Replacing the marbles with smaller ones, lead to different levels of *in vitro* RS_H for ground barley and ground maize, but not to a different ranking (data not shown). It seems that *in vivo* degradation of the cereal matrix is not only depending on the physical force applied by the GIT, but on other factors as well. As discussed in Chapter 3, we presume that both the protein rich endosperm as well as the cell wall material contribute to the observed discrepancies in predicted and actual RS_H values. Possibly, bacteria present in the upper GIT degrade cell wall material and make starch available for enzymatic hydrolysis by endogenous pig enzymes. The bacterial enzymes involved are likely to have a complex interaction with cell wall material, which seems to result in a more selective degradation of the cereal matrix than the physical degradation obtained with the *in vitro* assay.

THE EFFECT OF GASTRIC RETENTION ON PORTAL GLUCOSE APPEARANCE

The time it takes for glucose, originating from starch, to appear in the bloodstream depends on several aspects in addition to starch hydrolysis kinetics. One of the main aspects is the time it takes for starch to travel through the GIT until it is hydrolysed to glucose. This depends mainly on the gastric retention time of digesta, which is larger than the small intestine retention time (Chapter 4). Therefore, to fully understand the rate of starch digestion, it is important to understand which phenomena affect gastric retention time and how these, in turn, affect starch hydrolysis rates.

The relation between the gastric pH gradient and starch digestion kinetics

An important aspect in the role of the stomach on starch hydrolysis and glucose appearance kinetics, is the layer formation of digesta in the stomach. This layer formation and its effect on the pH distribution in the stomach was previously studied in depth by Bornhorst and colleagues (**Figure 7.7**)[29]. They observed that physical characteristics of digesta do not only affect the total gastric retention time, but also the mixing and pH of gastric digesta. For example, rigid feed components such as almonds causes the pH in the upper halve of the stomach to remain around pH 5 for 9 hours longer than soft feed components such as white rice. Gastric layering and the subsequent difference in pH gradient greatly affects starch digestion in continuously fed pigs, as both porcine salivary and gastric bacterial enzymes were found to be mainly active at a pH above 5 (Chapter 6).
Porcine salivary amylase hydrolyses insoluble starch into soluble starch break down products

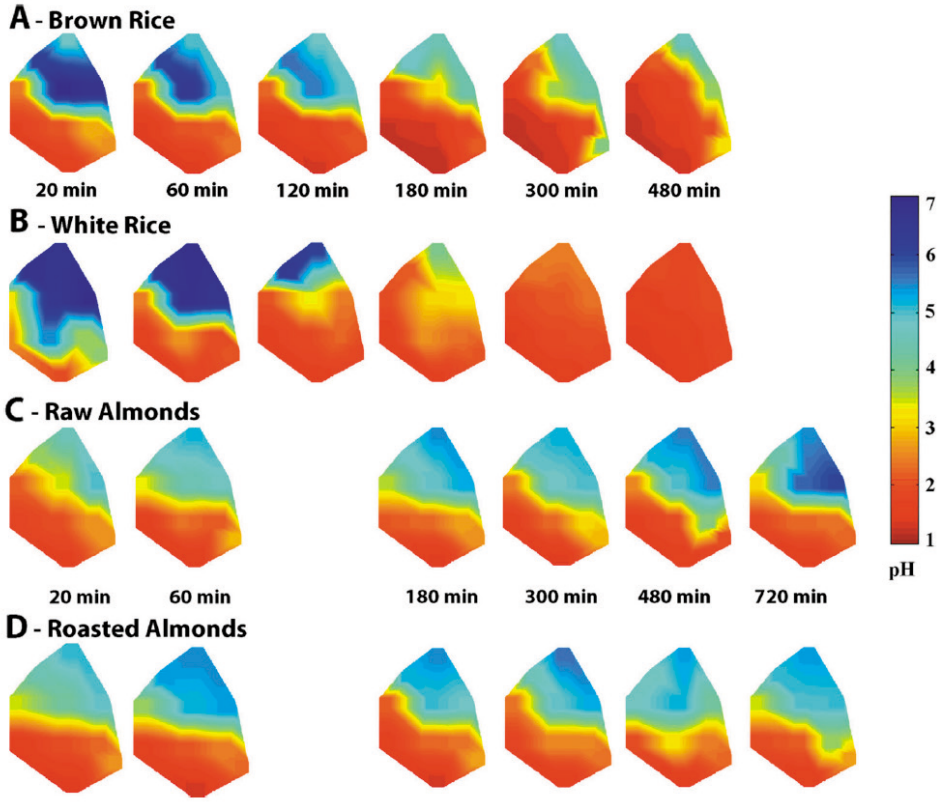

Figure 7.7. Colour maps showing intragastric pH values after consumption of either soft (A and B) or rigid (C and D) particle meals over a 480 to 720 min digestion period. Note that the digestion times shown for A and B are different from those shown for C and D. Each individual "stomach" represents the average of 6 pigs with 10 individual data points per pig. The top of each displayed stomach is connected to the oesophagus, whereas the bottom is connected to the small intestine. Reproduced with permission[29].

(Chapter 6). These soluble products, moving with the liquid phase of digesta, can appear more than one hour earlier in the small intestine (Chapter 4) and consequently contribute to a faster blood glucose response compared with insoluble starch. Also gastric bacteria can contribute to a faster blood glucose response when bacterial enzymes pre-digest starch and make starch granules more rapidly degradable by endogenous enzymes of the pig. The fraction of glucose that is actually consumed by bacteria will be converted into biomass and end products of glucose dissimilation pathways such as CO_2 and volatile fatty acids[30]. These products will not directly affect the rate of glucose appearance in the portal circulation, but they will contribute to a lower nett portal glucose recovery, as discussed in Chapter 6.

The relation between physical digesta properties and starch digestion kinetics

Digesta behaviour, such as retention time (Chapter 4) and mixing[29,31,32], are believed to depend greatly on physical properties. In turn, physical properties will affect gastric layering and the pH gradient in the stomach. Stomach digesta of pigs fed diets with isolated starch, as reported in this thesis, were visually comparable to stomach digesta of pigs fed brown rice by Bornhorst and colleagues[33]. Consequently, stomach digesta of pigs fed diets with isolated starches will likely have had a similar pH gradient as the pH gradient reported for pigs fed brown rice (**Figure 7.7**). Using this pH gradient as a basis for our dynamic *in vitro* stomach model, we found up to 10% hydrolysis of native starch using bacterial enzymes (Chapter 6). This fitted well with our own *in vivo* findings, but not necessarily with previous *in vivo* results. For example, in a previous study very limited signs of starch degradation were observed in the first meter of the small intestine of pigs[12], suggesting minor starch digestion in the stomach. Possibly, this difference in gastric digestion is caused by a different pH gradient in the stomach of pigs. In contrast to our study, pig in this previous study were fed a "western style meal", which contained comparable levels of starch (47.5%) as the diets in this thesis. However, these wester style meals were much higher in ingredients from animal origin (23.6% cooked red meat and 5% tallow) and much lower in fibrous feedstuffs from plant origin (4% wheat bran). The exact physical and rheological properties of digesta of pigs fed the western style meal are not known. However, it seems that differences in those properties, possible related to the low fibre content or high fat content, have led to a smaller contribution of gastric amylases on starch disappearance in pigs fed western style diets.

The relation between feeding regimes and starch digestion kinetics

In case of the continuously fed pigs described in Chapter 3 and 4 of this thesis, we assumed that the stomach was continuously filled for a considerable part. Consequently, every new meal ingested by the pig is likely to remain on top of the already present digesta, without complete mixing, as observed previously in pigs[32]. Consequently, in continuously fed pigs, each layer of digesta would move gradually from the proximal side of the stomach to the distal side. Because gastric acid is only secreted in the lumen of the stomach and mixing is

not complete[29], continuous feeding presumably creates large differences in pH of each layer, given that pigs are fed a diet that results in non-fluid stomach digesta. This might differ for pigs that are meal-fed. An empty stomach has a low pH (1.5-2.0)[31,34,35], thus the first part of an ingested meal may be easily decreased in its pH value. Subsequently ingested parts of a meal would fill the stomach to such an extent, that mixing will become more difficult. In turn, this will result in a low pH of digesta in the bottom of the stomach and a high pH in the top of the stomach, as reported by Bornhorst and colleagues[31]. For example, the pH in the lower part of the stomach is decreased within 20 minutes to a pH value below 5 , at which porcine saliva and bacterial enzymes become inactive (Chapter 6). However, if this same part of a meal would be ingested into a full stomach, the pH may easily remain at values at which porcine saliva and bacterial enzymes are active. Consequently, when pigs are meal-fed, the extent of gastric starch digestion is not homogeneous throughout the stomach, but will likely depend on the order of ingestion.

Positioning the englyst assay as tool for predicting portal glucose appearance kinetics

Ever since Englyst has introduced his *in vitro* characterization of starch digestion kinetics, there has been the need for an evaluation tool to understand and characterise *in vivo* starch digestion kinetics. For human studies, one approach is the recording of the extended glycaemic index (EGI), as suggested by Zhang and co-workers[36]. Typically, a slowly digestible starch is gradually hydrolysed *in vitro*, resulting in a slow but continuous glucose release. In the *in vivo* situation, a rapidly digestible starch results in a steep peak in plasma glucose concentration. Zhang and co-workers advocate that starch that is digested slowly *in vivo*, should theoretically result in a *lower* plasma glucose response than a rapid starch during this peak and a *higher* glucose response after this peak.

Animal models provide an excellent opportunity to test this hypothesis, as quite some research has been recorded and published on the net glucose responses in the portal vein over time after starch ingestion. To this end, I analysed the net glucose responses across studies that involved portal vein catheterised pigs, which were fed either a "slow starch" or a "rapid starch". A summary is given in **Table 7.3**, in which the time to peak is recorded for the rapid starch, in addition to significant differences in initial glucose response and extended glucose response. Surprisingly, in 13 out of 14 cases[2,15,37-41], feeding a slow starch did not result in a longer lasting net glucose flux in the portal vein compared with feeding a rapid starch. Only in one case, when feeding native pea starch[38], an actual prolonged net portal glucose recovery was observed, although others did not report this phenomenon upon feeding the same starch source[15,37]. This observation greatly challenges the relevance of the *in vitro* characterization system of Englyst and, consequently, the assumption that *in vitro* characterised slow starches are *in vivo* truly digested slower than rapid starches.

The *in vivo* and *in vitro* rates of starch digestion measured in this thesis provide new insights in

Table 7.3. Characteristics of portal vein glucose responses in pigs ingested a rapid versus a slow starch.

Rapid starch source	Slow starch source	Time to peak for rapid starch	Lower initial glucose response for slow starch?	Longer lasting glucose response for slow starch?	Source
Waxy sorghum	Non waxy sorghum	45 min	No	No	[42]
White bread	Native rice starch	50 min	**Yes**	No	[39]
White bread	Native pea starch	50 min	**Yes**	No	[39]
White bread	Native high amylose maize starch	50 min	**Yes**	No	[39]
Native waxy rice starch	Native rice starch	50- 60 min	**Yes**	No	[6,39]
Native waxy rice starch	Native pea starch	50- 60 min	**Yes**	No	[6,39]
Native waxy rice starch	Native high amylose maize starch	50-60 min	**Yes**	No	[6,39]
Wheat flour	Native potato and high amylose maize starch	60 min	**Yes**	No	[43]
Rye flakes	Native potato and high amylose maize starch	60 min	**Yes**	No	[43]
Gelatinized maize starch	Native maize starch	100 min	No	No	[5]
Native maize starch	Pea starch	2-6 hour	**Yes**	**Yes**	[40]
Sticky rice meal	Native high amylose maize starch	4-8 hour	**Yes**	No	[41]
Brown rice meal	Native high amylose maize starch	4-8 hour	**Yes**	No	[41]
Native maize starch and meal	Native high amylose maize starch	4-8 hour	**Yes**	No	[41]

this observation. To this end, the fractional rate of glucose release per minute was calculated for the fraction of RHS (0 to 20 min) and SHS (20 min to 120 min for *in vitro* and 20 min to the ileum for *in vivo*) for the diets described in this thesis. The *in vitro* digestion rates of native maize and barley starch were approximately 2% per minute and 0.5% per minute, respectively, for the RHS and SHS fractions. *In vivo*, however, the RHS fraction of isolated maize and barley starch disappeared from the small intestine at a rate of approximately 4% per minute, assuming no starch disappearance in the stomach. In contrast, the SHS fraction disappeared from the small intestine at around 0.2% per minute. The *in vivo* rates of starch disappearance of these slow starches, were almost similar to those of typical rapid starches, such as extruded barley or corn. This could explain why no difference in portal glucose response was observed in previous studies[2,15,37-41].

Typical sources of slow starches reported in **Table 7.3** were native starches such as non-waxy rice, pea, maize, and sorghums starch, or starches that were partly resistant, such as high amylose maize starch or potato starch. Comparing portal glucose response of partly resistant starches with non-resistant starches, we noticed that in addition to the initial lower glucose response, the total net glucose recovery was lower for resistant starches compared with non-resistant starches[15,37,39,41]. As found in our *in vivo* study, the RHS fraction of high amylose maize starch disappeared fairly rapidly from the small intestine (2% per min), whereas the SHS fraction disappeared much slower (0.3% per minute). Starches such as high amylose maize starch are typically considered partly resistant, but, according to the *in vitro* definition, also partly slowly digestible. However, our *in vivo* data indicates that the non-resistant fraction of those starches is actually fairly rapid digestible.

In conclusion, starch fractions quantified by the *in vitro* Englyst assay do not always predict the portal glucose response correctly. Based on this thesis, the difference in the *in vitro* and *in vivo* situation seems related to the initial rate of starch digestion, which was higher *in vivo* than *in vitro*. Gastric starch digestion or pre-digestion seem to contribute to the more rapid initial starch digestion *in vivo* and seems to be a key factor in an accurate prediction of starch digestion rates.

Future perspectives

Measuring starch digestion kinetics *in vivo*

As discussed in the beginning of this chapter, various measurement techniques can be employed to quantify digested starch. The research presented in this thesis is amongst the first published works on *in vivo* hydrolysis kinetics of starch, which shows how large the difference can be between hydrolysed and disappeared starch, especially in the proximal small intestine. Consequently, the method used to measure starch "digestion" can greatly affect someone's definition of digested starch.

In the introduction of this thesis, I have brought up that the energetic and nutritional value of starch does not only depend on the extent of starch digestibility, but also on the effect that

starch has on the utilization of other nutrients. Currently, much remains unknown about this complex interplay. A more accurate measurement of starch hydrolysis rate and the presence of breakdown products throughout the small intestine, using the slaughter technique, may provide new insights in this matter.

Gastric (pre-)digestion

The research presented in this thesis has shown that bacterial enzymes have the potential to degrade a considerable fraction of starch in the stomach of pigs. The extent, however, depends greatly on the pH gradient and retention time of digesta in the stomach. We obtained valuable new insights in the gastric retention time and rheological properties of gastric digesta: the concentration of large particles negatively affects the deformability of digesta in continuously fed pigs and, in turn, this decrease in deformability increases gastric retention times. However, predicting the pH gradient and retention time of digesta in the stomach is still hampered by several factors:
Firstly, we were not able to predict physical digesta properties based on dietary properties. Most likely, the rheological behaviour of digesta is determined by the physical properties of large particles, which accumulate in the stomach. I hypothesize that differences in deformability and water holding capacity of large particles are the main factors that determine rheological properties of stomach digesta. Therefore, more research on the relation between the physical properties of each particle size fraction in diets and those in stomach digesta, may enable the future prediction of mixing behaviour and retention time of stomach digesta. Secondly, we observed a different ranking in gastric emptying times when diets were fed continuously or in meals. At the moment, the relation between feeding regimes and rheological properties of stomach digesta remains unclear. Consequently, more insights in the driving forces of gastric layering across different feeding regimes is required, which would allow for a more accurate prediction of gastric starch digestion in the future.

The future of *in vitro* starch digestion assays

In vitro analyses, usually based on Englyst's assay, are widely used to predict starch digestion kinetics. However, this analysis does not predict *in vivo* starch digestion rates correctly, for which we identified several potential causes and points of attention in future research. First, gastric digestion is not accounted for in current *in vitro* systems at all, which may cause an overestimation of the total amount of digestible starch. Secondly, gastric pre-digestion is not taken into consideration, which might lead to an underestimation of the amount of rapidly digestible starch. However, as indicated above, more research on the relation between properties of gastric digesta and bacterial starch degradation is required first, before this implementation can be made. Thirdly, *in vitro* analyses generally do not take the, possible synergistic, effect of brush border enzymes and pancreatic α-amylase into account. Finally, current *in vitro* methods fail to accurately predict the extent of starch digestion for starches present in their cereal matrix. Bacterial enzymes present in the GIT seem more selective in the

breakdown of the cereal matrix compared with current *in vitro* methods. Consequently, each cereal matrix is considered equal based on *in vitro* analysis, whereas the effect of the cereal matrix on *in vivo* starch digestibility varies between cereals from different sources. Possible, the endosperm structure of cereals and its effect on starch digestibility is also affected by the cereal strain or even seasonal conditions.

Take-home messages of this thesis

This thesis provides new insight in the *in vivo* rate of starch digestion and its relation with digesta passage behaviour in the upper GIT.

- The effects of starch properties on *in vitro* starch digestion kinetics depend on the botanic origin of the starch.
- The initial rate of starch digestion in pigs is higher than its current *in vitro* predictions.
- The discrepancy between the initial rate of *in vivo* and *in vitro* starch digestion appears related to: Gastric hydrolysis of starch by salivary and bacterial amylases and a synergistic action of brush border enzymes and pancreatic α-amylase in the proximal small intestine.
- The extent of starch hydrolysis by gastric bacteria depends on the physical properties, and consequently the pH gradient, of gastric digesta.
- The ^{13}C breath test is a promising, non-invasive tool to measure gastric emptying in pigs and revealed a biphasic emptying pattern after meal feeding.
- Starch digestibility measured in the ileum overestimates the true level of glucose availability due to the utilization of starch by gastric bacteria.
- Starch digestion kinetics is heavily affected by the cereal matrix, making isolated starches of limited value for common feed evaluation purposes.
- Part of starch is truly resistant to *in vivo* degradation, either due to its embedding in the plant matrix or due to a resistant granular structure.
- Current *in vitro* methods that include standardized physical disruption of the cereal matrix, fail to predict the matrix effect on *in vivo* starch digestion.
- The truly resistant fraction of starch does not depend on the digestible fraction: typical sources of slow starch, such as high amylose starch, actually seem partly rapidly digestible and partly resistant *in vivo*

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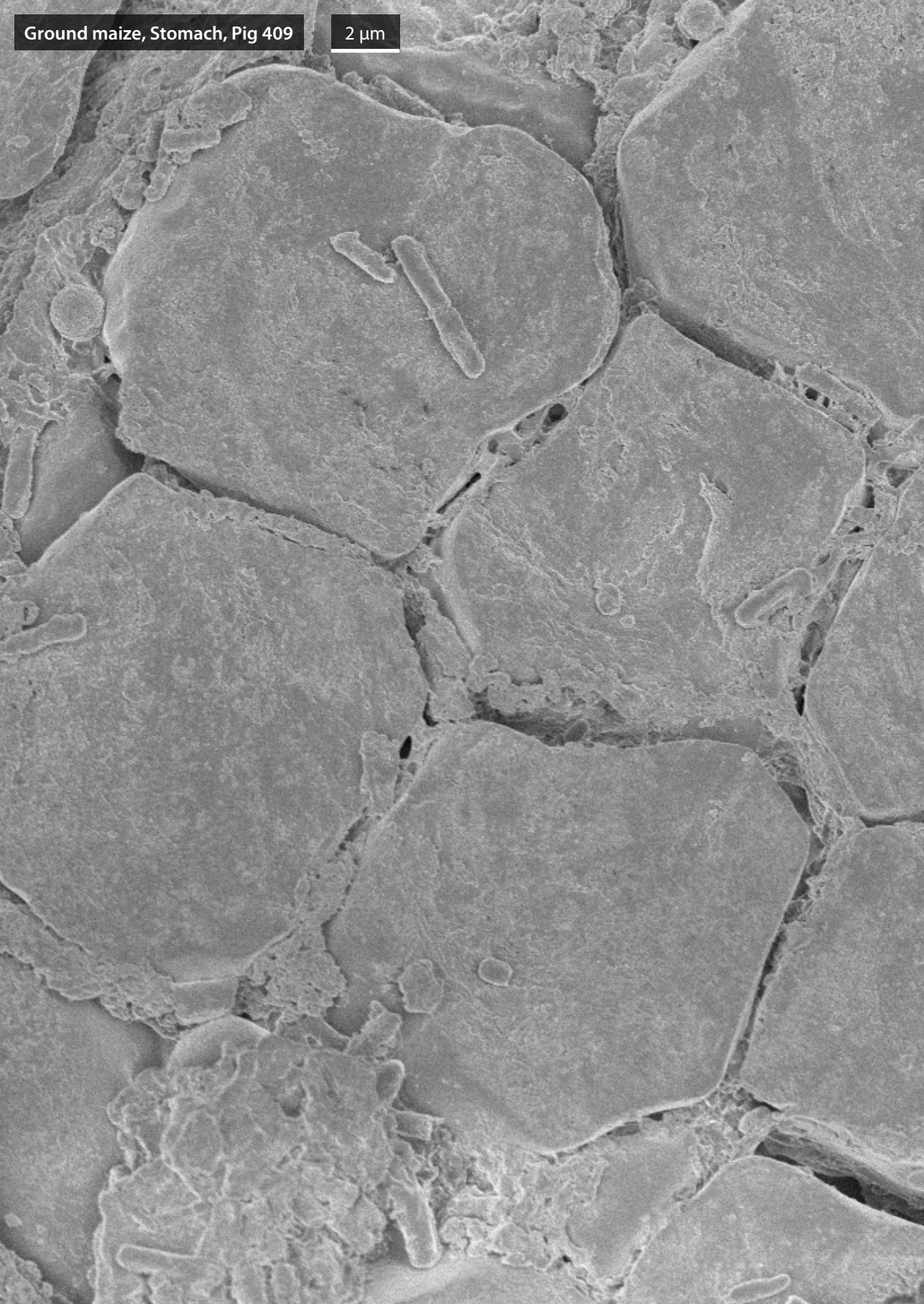
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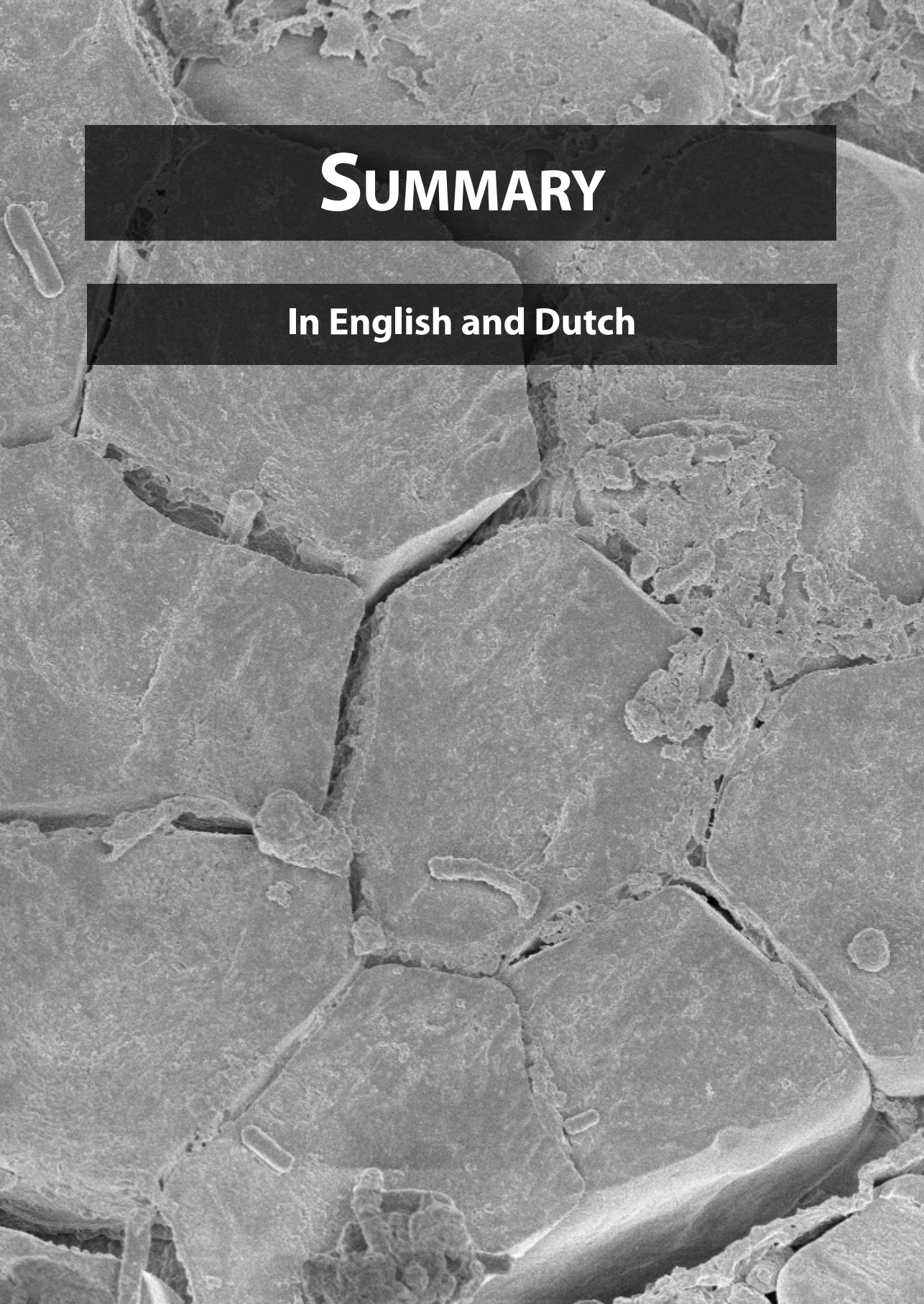


Summary

In English and Dutch

Summary

The main aim of this thesis was to quantify the contribution of intrinsic starch structure, feed processing, and digesta passage behaviour on the kinetics and mechanisms of starch digestion in the upper gastrointestinal tract (GIT) of pigs.

Research in **Chapter 2** focused on the relation between *in vitro* starch hydrolysis rates and structural properties of starch. The main conclusion of this chapter was that the structural properties of starch that affect *in vitro* hydrolysis kinetics, depend greatly on the botanic source of the starch. Across botanic sources, increased concentrations of A-type crystalline structure and short amylopectin side-chains increased hydrolysis rates. Other properties explained additional variation in *in vitro* hydrolysis kinetics within botanic sources. Within maize starches, for example, an increased *in vitro* hydrolysis rate of starch was related to a decreased amylose content and an increased number of pores per starch granule.

Chapter 3 describes the translation of the relation between structural starch properties and *in vitro* starch hydrolysis, to *in vivo* starch hydrolysis in growing pigs. Consistent with our *in vitro* findings, an increased amylose content negatively affected the digestion rate of maize starches in pigs. In addition to starch structure, the cereal matrix and processing conditions of feedstuffs affected *in vivo* starch hydrolysis. Starch originating from ground barley was fully digestible in the small intestine of pigs, whereas 16% of starch ingested as ground maize was resistant to digestion. Extrusion increased digestibility in proximal and distal parts of the small intestine. From Chapter 3 we concluded furthermore that a major part of starch disappears from the GIT in the proximal small intestine. Starch digestion coefficients in the proximal small intestine varied from 0.16 to 0.63 across starches from different botanic sources, in processed or native form. In addition, a substantial amount of starch (up to 25% of ingested starch) was present as soluble maltodextrins in the proximal small intestine.

In **Chapter 4** of this thesis, the gastric and small intestine retention time of starch rich diets in pigs is presented after pigs were fed continuously. From this research we concluded that digesta mean retention times (MRT) in pigs were longer for the stomach (129 to 225 min for solids) than for the small intestine (86 to 124 min for solids). In addition, liquid digesta remained around 1 hour shorter in the stomach compared to solid digesta. The MRT of solid digesta in the stomach was decreased by extrusion and was positively correlated with the shear stress required to deform digesta. Against our expectations, the strongest correlation between MRT and digesta properties in the stomach was not identified between digesta MRT and shear stress, but between MRT and the so-called saturation ratio (SR). The SR is the actual amount of water in stomach digesta as a fraction of the theoretical maximum held by digesta dry matter present in the stomach, based on its water holding capacity. The predictability of SR from feed properties was hampered by the accumulation of large particles in the stomach. Stomach digesta of pigs fed diets with extruded cereals were characterized by the presence of maltodextrins. The presence of these break down products indicate that the starch network, responsible for a high water holding capacity in the diet, does not remain intact in the stomach of pigs. Indeed, for pigs fed diets with extruded cereals, we observed a high SR of stomach digesta. For small intestine digesta, no correlation was

identified between MRT and rheological properties.
Small intestine digesta retention times presented in **Chapter 4** enabled the calculation of starch hydrolysis kinetics, which is presented in **Chapter 3**. Starch hydrolysis in the proximal small intestine was underestimated by standard *in vitro* approaches, whereas the amount of starch resistant to hydrolysis by endogenous enzymes of the pig exceeded our *in vitro* predictions. In addition, glucose release from slowly digestible starch is less gradual than predicted from *in vitro* analysis. Based on the large underestimation of starch disappearance in the proximal small intestine, we hypothesized that the role of the stomach on *in vivo* starch disappearances might currently be underestimated.
In **Chapter 5**, the pattern of gastric emptying of pigs is described using the ^{13}C breath test, after feeding the same diets as described in the previous chapter. The ^{13}C breath test revealed a biphasic pattern of gastric emptying after meal feeding and we estimated that around 60% of the meal was emptied from the stomach during the first phase. The first peak in gastric emptying of solids reached its maximum within two hours after feeding in all cases. For digesta liquids, this peak was reached 36 minutes earlier for pigs fed ground barley, compared with pigs fed diets containing isolated barley starch. The second peak in gastric emptying of solids was reached later for pigs fed ground barley (5.9 hours after feeding), compared with pigs fed extruded barley (4.5 hours after feeding) and pigs fed diets containing isolated barley starch (4.8 hours after feeding). Diets ranked similar in the time to reach the second peak as they did in gastric MRT when pigs were fed continuously (chapter 4). However, the time needed for a diet to reach the first peak in gastric emptying had a ranking that deviated from MRT-based ranking in continuously fed pigs.
In **Chapter 6**, our research towards the contribution of gastric starch degradation on *in vivo* starch digestion kinetics is described. Current *in vitro* systems assume that starch is left untouched in the stomach of pigs. Upon analysis of stomach digesta of pigs, however, we identified soluble break down products of starch. In addition, we found evidence of granular starch degradation by bacteria in the stomach of pigs with electron microscopy. To this end, we designed a stomach model in which the pH was step-wise decreased from 6.5 to 2.0 in 225 minutes. Bacterial enzymes, extracted from stomach digesta of pigs, hydrolysed up to 29% of starch under these conditions. In addition, porcine salivary α-amylase, which has an optimum pH around 7.8, degraded up to 10% of gelatinized starch under stomach conditions. However, the effect of salivary α-amylase on native starch was negligible. In contrast, bacterial enzymes were active on both native and gelatinized starch.
Chapter 7 provides a general discussion on starch digestion kinetics throughout the upper GIT of the pig, combining the results described in this thesis with existing literature. Seemingly, starch fractions quantified by the *in vitro* Englyst assay do not always predict the *in vivo* portal glucose response correctly. Based on the research presented in this thesis, the difference in the *in vitro* and *in vivo* situation seems related to the initial rate of starch digestion. Gastric starch digestion or pre-digestion by both porcine saliva and gastric bacteria seem to contribute to the more rapid initial starch digestion *in vivo*. Digesta passage and mixing behaviour, especially in the stomach of pigs, is currently an underestimated aspect in *in vitro* methods, but seems to be a key factor in an accurate prediction of starch digestion rates.

Samenvatting

Eerder onderzoek heeft uitgewezen dat de snelheid waarmee zetmeel verteerd wordt in varkens invloed kan hebben op de benutting van zetmeel en andere nutriënten, en daarmee op de energetische en nutritionele waarde van varkensvoer. Het belangrijkste doel van dit proefschrift was om meer inzicht te krijgen in het verband tussen de snelheid van zetmeelvertering en de eigenschappen van zetmeel, de toegepaste procestechnologie, en het passagegedrag van maag- en darminhoud.

Hoofdstuk 1 van dit proefschrift bevat een algemene introductie en beschrijft de bestaande kennis op het gebied van zetmeelvertering in varkens, de kennis die volgens mij ontbrak, en de doelen die ik vervolgens gesteld hebben om deze kennis in te vullen. Om dit te bereiken heb ik, met hulp van mijn collega's van Wageningen Universiteit en Agrifirm, een aantal experimenten uitgevoerd, zowel in varkens (*in vivo*) als buiten het dier (*in vitro*), die beschreven zijn in de volgende vijf hoofdstukken van dit proefschrift.

Het onderzoek in **Hoofdstuk 2** was gericht op het verband tussen de *in vitro* verteringssnelheid en intrinsieke eigenschappen van zetmeel. De belangrijkste conclusie van dit hoofdstuk was dat de structurele eigenschappen van zetmeel die verteringssnelheid beïnvloeden sterk afhangen van de botanische bron van het zetmeel. Voor zetmelen van verschillende plantensoorten geld dat een A-type kristalstructuur en korte ketens van amylopectine gerelateerd zijn aan een snellere zetmeelvertering. Binnen één botanische bron bleken andere zetmeel eigenschappen een aanvullende rol te spelen. Bij zetmelen afkomstig van maïs, bijvoorbeeld, kon een snelle zetmeelvertering verklaard worden door een afname in het amylosegehalte en een toename in het aantal poriën op een zetmeelkorrel.

Met de studie die beschreven is in **Hoofdstuk 3** hebben we geprobeerd het *in vitro* verband tussen zetmeel eigenschappen en verteringssnelheden te vertalen naar de *in vivo* situatie. Dit hebben we gedaan door negen soorten voer te produceren, verschillend in de *botanische herkomst* van zetmeel en de toegepaste *procestechnologie*. We hebben hierbij gekozen voor zetmeel afkomstig van gerst, maïs, en een gemodificeerde variant van maïs met een verhoogd amylosegehalte. Alle drie deze zetmelen zijn als geïsoleerd poeder, gemalen graan, en geëxtrudeerd graan in een experimenteel voer verwerkt. Zoals verwacht zorgde een verhoogd amylosegehalte van maïszetmelen inderdaad voor een lagere afbreekbaarheid van zetmeel in varkens. Daarnaast werd de vertering van zetmeel beïnvloed door de graanmatrix en de procestechnologie die gebruikt was om het voer te maken. Zetmeel in gemalen gerst, bijvoorbeeld, was volledig verteerbaar, terwijl zetmeel in gemalen maïs voor 16% onverteerd bleef in de dunne darm. Extrusie maakte zetmelen een stuk beter verteerbaar, zowel voorin als achterin de dunne darm. Opvallend was ook de forse hoeveelheid zetmeel, 16 tot 63%, die in het eerste deel van de dunne darm was verteerd. Verder bleek een aanzienlijk deel van het zetmeel (tot 25% van het opgenomen zetmeel) aanwezig te zijn als oplosbare afbraakproducten in het eerste deel van de dunne darm.

De snelheid van zetmeelvertering in het dier wordt niet alleen bepaald door de snelheid waarmee enzymen zetmeel afbreken, maar ook door de snelheid waarmee het zetmeel door

het maagdarm-kanaal van een dier wordt getransporteerd. Deze zgn. digestapassagesnelheid is beschreven in **Hoofdstuk 4**. We hebben gevonden dat het onoplosbare deel van maag- of darminhoud doorgaans langer in de maag van varkens blijft (variërend van 129 tot 225 minuten) dan in de dunne darm (86 tot 124 minuten). Het oplosbare deel van de maaginhoud blijft ongeveer een uur korter in de maag dan het onoplosbare deel. In de dunne darm had juist de onoplosbare fractie een kortere passagetijd dan de oplosbare fractie. Extrusie van graan zorgde voor een kortere verblijftijd van voer in de maag en dit bleek gerelateerd te zijn aan de reologische eigenschappen van de maaginhoud. Maaginhoud die makkelijker vervormbaar is, passeert de maag sneller. Tegen onze verwachting in verklaarde deze reologische eigenschap niet de meeste variatie in de passagesnelheid van maaginhoud. De meeste variatie werd namelijk verklaard door de zgn. verzadigingsfactor van de maaginhoud. Als er minder water in de maag aanwezig is dan de hoeveelheid water die gebonden kan worden door de maaginhoud, passeert het de maag langzamer dan wanneer er water in overvloed aanwezig is. Deze factor bleek echter moeilijk te voorspellen uit eigenschappen van het voer. Wanneer het voer in de maag van het varken verblijft, hopen grootte deeltjes zich op, waardoor de fysische eigenschappen van de maaginhoud anders zijn dan die van het voer. Daarnaast vonden we oplosbare afbraakproducten van zetmeel in de maag van varkens die geëxtrudeerd voer hadden gegeten. Deze afbraakproducten duiden erop dat er in de maag afbraak plaatsvind van het zetmeelnetwerk, wat normaal gesproken voor een hoog waterhoudend vermogen zorgt in voer dat een hittebehandeling heeft ondergaan. Voor dieren die we geëxtrudeerd voer hadden gevoerd, vonden we dan ook een hoge verzadigingsfactor van de maaginhoud ten opzichte van de verzadigingsfactor van het voer.

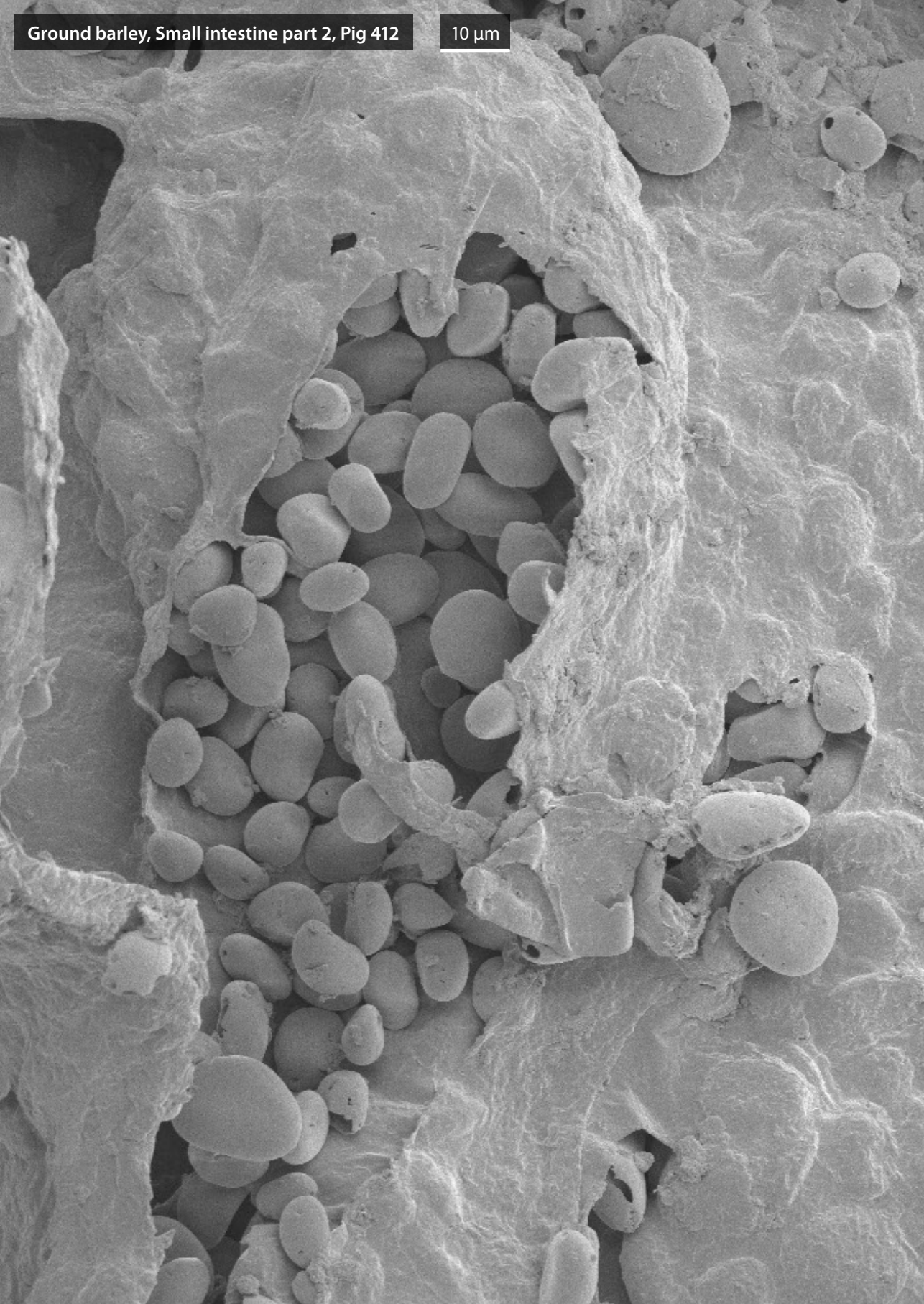
Naast de passagesnelheid van maaginhoud hebben we ook de passagesnelheid van darminhoud gemeten en gerapporteerd in **Hoofdstuk 4**. Hiermee hebben we iets kunnen berekenen wat nooit eerder is gedaan, namelijk de snelheid van zetmeelvertering, in procent per minuut, in het varken. Dit konden we vervolgens vergelijken met de eerder gemeten *in vitro* verteringssnelheid, en beide zijn beschreven in **Hoofdstuk 3**. Opmerkelijk was dat de *in vivo* verteringssnelheid van zetmeel veel hoger was dan die *in vitro* gemeten was: in de dunne darm van het varken was 35% van het zetmeel verteerd binnen 5 minuten, terwijl dit slechts 14% was met onze *in vitro* methode. Dit grote verschil deed ons vermoeden dat er, in het dier, een rol voor de maag zou kunnen zijn op zetmeelvertering. Deze potentiële rol voor de maag wordt tot nu toe onderschat in *in vitro* methodes.

De studies die beschreven zijn in hoofdstuk 3 en 4 zijn uitgevoerd met dieren die continu gevoerd zijn. Daartegenover hebben we de maaglediging gemeten van dieren die in grotere maaltijden zijn gevoerd, wat beschreven staat in **Hoofdstuk 5**. In deze studie hebben we de ^{13}C ademtest gebruikt om maaglediging van varkens te meten die ook de negen voeders uit hoofdstuk 3 en 4 hebben gekregen. De ^{13}C ademtest is een niet-invasieve methode, waarbij een gelabelde markeerstof terug gemeten kan worden in de adem van een varken, nadat deze markeerstof de maag heeft gepasseerd. Met deze test hebben we gevonden dat de maag in twee fases werd geledigd, waarbij ongeveer 60% van de maaltijd in de eerste fase werd geleegd. De piek in deze eerste fase werd doorgaans binnen twee uur na voeren bereikt

voor het onoplosbare deel van de maaginhoud. Lediging van het oplosbare deel van de maaginhoud werd beïnvloed door de vorm waarin het zetmeel verstrekt was: varkens die gemalen gerst waren gevoerd vertoonde 36 minuten eerder een piek in maaglediging dan varkens die geïsoleerd gerstenzetmeel waren gevoerd. De tweede piek in maaglediging van de onoplosbare maaginhoud verscheen sneller voor dieren die we gemalen gerst hadden gevoerd (5.9 uur na voeren) dan voor dieren die we geëxtrudeerd gerst (4.5 uur na voeren) of geïsoleerd gerstenzetmeel (4.8 uur na voeren) hadden gevoerd. De volgorde waarin voeders deze tweede piek bereikten, was vergelijkbaar met volgorde in verblijftijd van voer in de maag zoals gerapporteerd in hoofdstuk 4. De volgorde waarin voeders de eerste piek bereikten, daarentegen, was niet gecorreleerd aan de verblijftijd van maaginhoud zoals beschreven in hoofdstuk 4.

Hoofdstuk 6 beschrijft ons onderzoek naar de rol van de maag op zetmeelvertering. We hebben eerst de maaginhoud van varkens onderzocht, waarin we oplosbare afbraakproducten van zetmeel vonden. Daarnaast hebben we visueel bewijs gevonden, middels foto's gemaakt met een elektronen microscoop, voor de afbraak van zetmeelkorrels door bacteriën in de maag. Om de bijdrage van deze bacteriën aan zetmeelafbraak te kwantificeren, hebben we bacteriële enzymen geëxtraheerd uit maaginhoud. Die enzymen hebben we vervolgens aan verschillende soorten voer toegevoegd in een dynamisch *in vitro* maagmodel, waarin we in 225 minuten stapsgewijs de pH verlaagd hebben van 6.5 naar 2.0. Tijdens deze incubatie werd tot 29% van het zetmeel afgebroken door het enzymextract dat verkregen was uit de maag. Om de omzetting door bacteriën te onderscheiden van de omzetting door speekselamylase, wat mogelijk actief blijft in de maag, hebben we dezelfde incubatie uitgevoerd met speeksel van varkens. We hebben gevonden dat dit enzym een optimum pH van 7.8 heeft en ongeveer 10% van het zetmeel afkomstig uit geëxtrudeerd gerst om kan zetten tijdens de incubatie onder maagcondities. Hieruit hebben we geconcludeerd dat de bijdrage van bacteriële enzymen op zetmeelafbraak groter lijkt te zijn dan dat van speeksel, met name op voeders die geen hitte behandeling hebben ondergaan.

Hoofdstuk 7 bevat een algemene discussie over de snelheid van zetmeelvertering in varkens waarin ik onze bevindingen in perspectief plaats van de bestaande literatuur. De belangrijkste bijdrage van het werk in dit proefschrift, is de vertaalslag van *in vitro* zetmeelvertering naar de *in vivo* situatie. In dit hoofdstuk leg ik uit waarom ik denk dat de vertering of mogelijke voorvertering van zetmeel in de maag verantwoordelijk is voor het grote verschil in initiële zetmeel afbraaksnelheid in de dunne darm van het varken ten opzichte van het *in vitro* model. De rol van de maag is sterk afhankelijk is van de fysische eigenschappen van de maaginhoud en is een onderschat aspect in de huidige *in vitro* modellen, maar is volgens mij een essentiële factor in een goede voorspelling van de zetmeel verteringssnelheid.


Ground barley, Small intestine part 2, Pig 412
10 µm

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Zetmeel, dat is inmiddels toch al volledig uitgekauwd in de wetenschap? In de zomer van 2014 hoorde ik dat ze daar in de diervoedingsindustrie heel anders over dachten. **Jean-Paul**, bedankt dat je me (in tweede instantie) overtuigd hebt om een PhD onderzoek te starten. Ik bewonder jouw drive om de passie voor wetenschap door te geven aan de nieuwe generatie. Ik zou er geen seconde over twijfelen om alle vier de jaren opnieuw te doen!
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in ons technologisch team!
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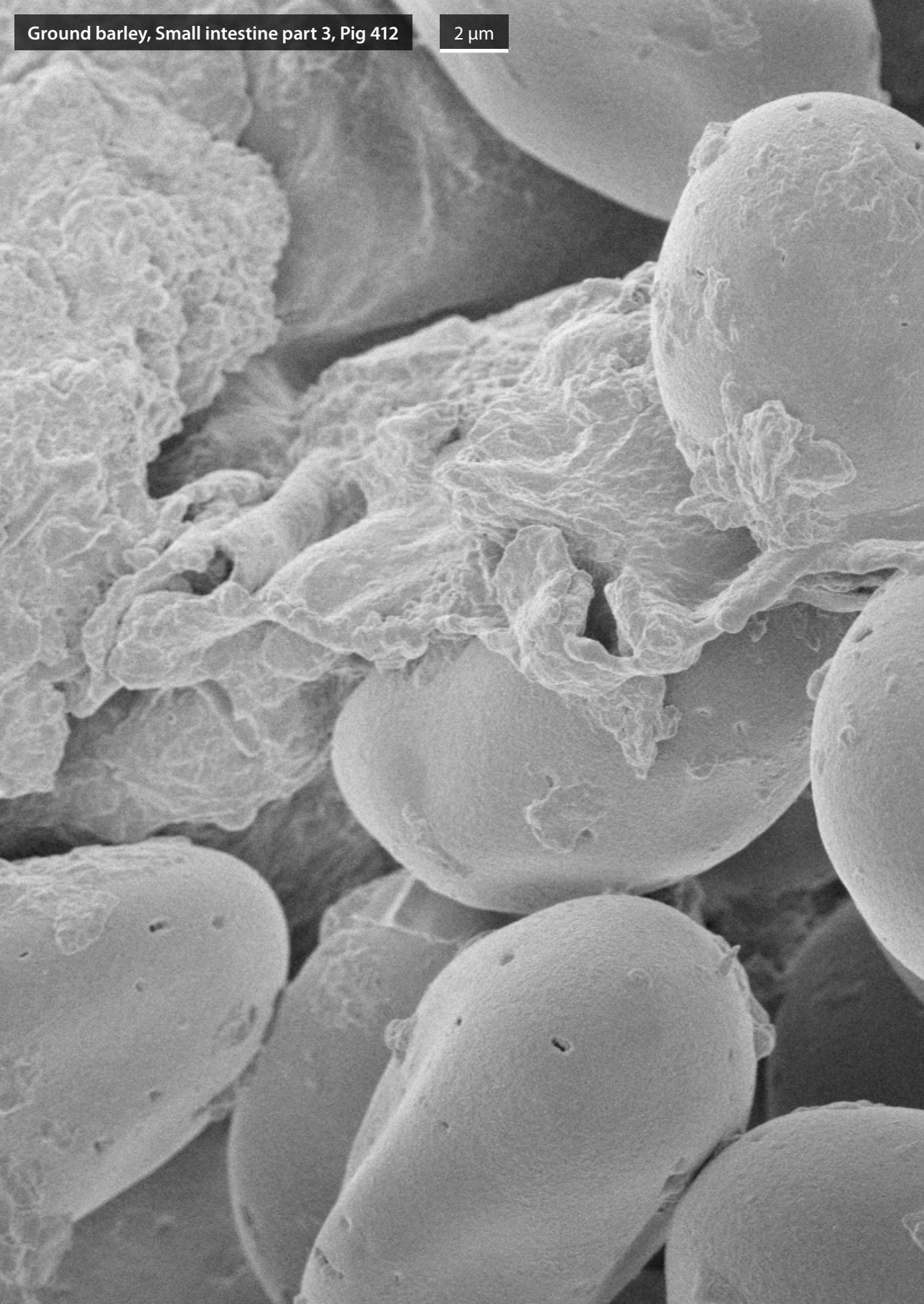
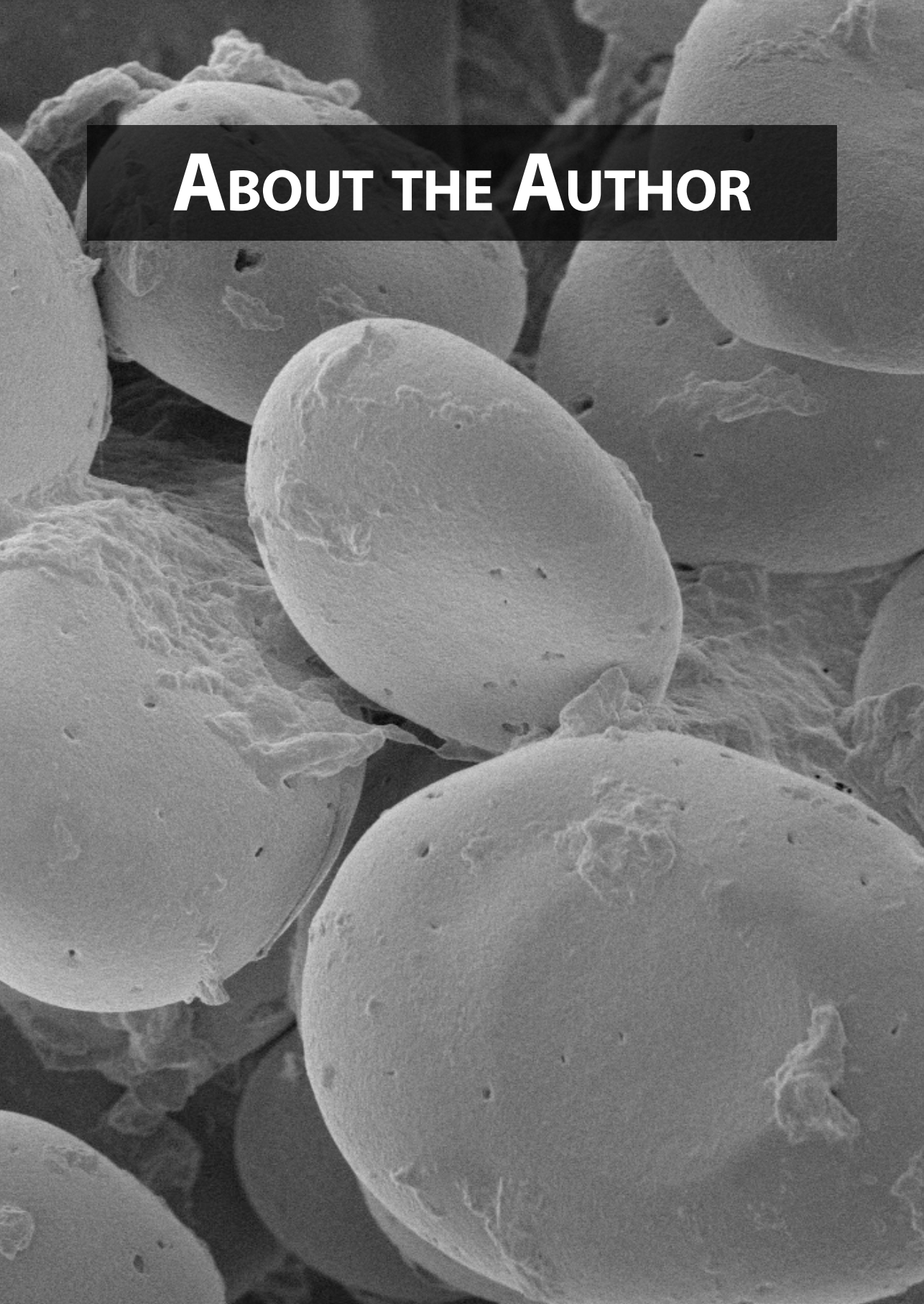

Ground barley, Small intestine part 3, Pig 412
2 µm



About the Author

Curriculum Vitae

Bianca Maria Johanna Martens was born on June 15th 1991 in Breda, The Netherlands. After graduating from high school (Markenhage college, Breda, The Netherlands) in 2009 she started the bachelor study Food Technology at Wageningen University. In 2012 she continued her studies at Wageningen University with the master Food Ingredient Functionality. In 2014 she finalized her MSc thesis entitled "*in vitro* prenylation of isoflavonoids with microbial prenyltransferases" and subsequently started an internship at Friesland Campina in Wageningen, The Netherlands. The same year she obtained her MSc degree and started as researcher biochemistry at Agrifirm Innovation Centre. Within Royal Agrifirm Group, she obtained the opportunity to work as PhD candidate at the Animal Nutrition Group and Laboratory of Food Chemistry, under the supervision of Prof. dr. ir. Walter Gerrits and Prof. dr. Henk Schols. The results of her PhD research are presented in this thesis. Bianca continues to work at the R&D department of Royal Agrifirm Group as researcher technology.

Contact: b.martens@agrifirm.com, bianca-martens@hotmail.com

List of publications

Martens BMJ, Bruininx EMAM, Gerrits WJJ, Schols HA (2019) The importance of gastric amylases in starch digestion kinetics in pigs. *Manuscript submitted for publication*.

Martens BMJ, Noorloos M, Schols HA, Bruininx EMAM, Gerrits WJJ (2019) Whole digesta properties as influenced by feed processing explain variation in gastrointestinal transit times in pigs. *Manuscript submitted for publication*.

Martens BMJ, Flécher T, de Vries S, Schols HA, Bruininx EMAM, Gerrits WJJ (2019) Starch digestion kinetics and mechanisms in growing pigs fed processed and native cereal based diets. *Br J Nutr.* (in press)

Martens BMJ, Gerrits WJJ, Bruininx EMAM, Schols HA (2018) Amylopectin structure and crystallinity explains variation in digestion kinetics of starches across botanic sources in an in vitro pig model. *J Anim Sci Biotechnol* **9**, 91

Araya-Cloutier C, **Martens BMJ**, Schaftenaar G, Leipoldt F, Gruppen H, Vincken, JP (2017) Structural basis for non-genuine phenolic acceptor substrate specificity of Streptomyces roseochromogenes prenyltransferase CloQ from the ABBA/PT-barrel superfamily. *PloS one* **12**, e0174665

Overview of completed training activities

Discipline specific activities	
Courses	
Pig feed in practice	Wageningen academy, Wageningen, 2014
Advanced food analysis	VLAG, Wageningen, 2015
Food and biorefinery enzymology	VLAG, Wageningen, 2015
Advances in feed evaluation science[a]	Wageningen academy, Wageningen, 2015
Conferences and seminars	
Starch round table	RUG and WUR, wageningen, 2014
Digestive physiology of pigs	DPP, Kliczków, 2015
Quality of protein in animal diets	WIAS, Wageningen, 2015
Nutrient requirements and animal health	ANU, WIAS, & WLR, Wageningen, 2015
Starch round table[a]	RUG and UDL, Lille, 2016
Digestive physiology of pigs[a,b]	DPP, Brisbane, 2018
Yearly CCC symposium[a,b]	CCC, Groningen, 2014-2018
General courses	
Vlag PhD introduction week	VLAG, Baarlo, 2015
Mobilising your scientific network	WSG, Wageningen, 2015
Effective behaviour in your professional surroundings	WSG, Wageningen, 2015
Scientific writing	WGS, Wageningen, 2016
Effectively influencing	Yearth Academy, Apeldoorn, 2016
Working with insights profiles	The insights Group, Apeldoorn, 2018
Optionals	
Research proposal preparation	FCH, Wageningen, 2014-2015
PhD study trip to Japan[a,b,c]	FCH, 2016
Laboratory Animal Science Course	WUR, Wageningen, 2016
PhD study trip to Italy and Austria[a,b]	FCH, 2018
BSc/MSc thesis student presentations	ANU & FCH, Wageningen, 2014-2019
PhD presentations	ANU & FCH, Wageningen, 2014-2019

[a] Oral presentation, [b] Poster presentation, [c] Organising committee

Abreviations: ANU, Animal Nutrition Group, Wageningen University, CCC, Carbohydrate Competence Center. DPP, Digestive Physiology of pigs. FCH, Laboratory of Food Chemistry, Wageningen University. RUG, University of Groningen, UDL, University of Lille. VLAG, Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Sciences. WGS, Wageningen Graduate Schools. WIAS, Wageningen Institute of Animal Sciences. WLR. Wageningen Livestock Research. WUR, Wageningen University & Research.

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