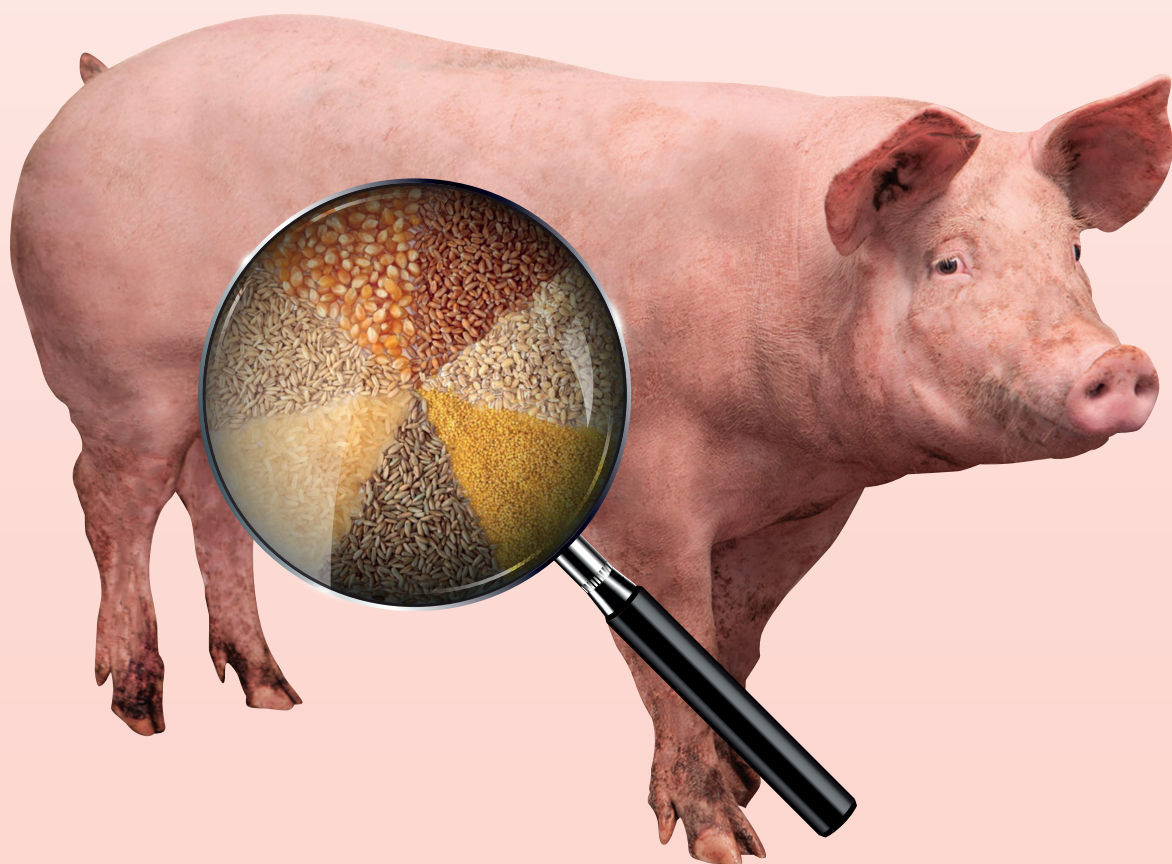# Nutrient yield from starch in pigs

Consequences for energy balance and meal patterns

Rik J.J. van Erp

Propositions

1. Under *ad libitum* feeding conditions, net energy values of digestible starch and resistant starch are similar.
    (this thesis)

2. The pig's meal pattern is an intrinsic characteristic that is difficult to influence by nutrition.
    (this thesis)

3. Data science should be a specialization within each scientific discipline, rather than a discipline itself.

4. Research in a company cannot reach its full potential without the support of an effective marketing strategy.

5. All responses on social media should be private.

6. In contrast to football, individualism cannot exist in handball.

Propositions belonging to the thesis, entitled

Nutrient yield from starch in pigs
Consequences for energy balance and meal patterns

Rik van Erp
Wageningen, 4 October 2019

Nutrient yield from starch in pigs

Consequences for energy balance and meal patterns

Rik J.J. van Erp

Thesis committee

Promotors
Prof. Dr W.J.J Gerrits
Personal chair Animal Nutrition Group
Wageningen University & Research

Prof. Dr L.A. Den Hartog
Special professor Animal Nutrition in a Circular Economy
Wageningen University & Research
R&D director Nutreco
Nutreco, Amersfoort

Co-promotors
Dr S. de Vries
Assistant professor, Animal Nutrition Group
Wageningen University & Research

Prof. Dr T.A.T.G. van Kempen
Adjunct professor, Animal Science
North Carolina State University, USA
Discovery lead, Trouw Nutrition
Nutreco, Amersfoort

Other members
Prof. Dr J. Keijer, Wageningen University & Research
Dr E. Labussière, INRA, Rennes, France
Prof. Dr K. Venema, Maastricht University, Maastricht
Prof. Dr K.E.B. Knudsen, Aarhus University, Denmark

Nutrient yield from starch in pigs

Consequences for energy balance and meal patterns

Rik J.J. van Erp

Table of contents

Chapter 1

General Introduction

1.1 Background

The world population has doubled since the early 1960s, and is predicted to grow well beyond 2050 [1]. To adequately feed the predicted population of 2050, food production must increase substantially [2]. Consequently, competition for resources that are used for production of ingredients used in human food and animal feed will increase. To overcome future scarcity in feed ingredients for animal feed, the demand for efficient utilization of presently used feed ingredients and for new alternative feed ingredients grows. Currently, approximately 40% of human calorie supply comes from rice, maize, and wheat [3]; starch-rich ingredients that are also substantially used in animal feed. Starch is the major dietary energy source for pigs, and takes up 40 to 55% of a pig diet on dry matter basis [4]. Most of the starch ingested is digested into glucose by host-enzymes. Starch that remains undigested serves as a substrate for microbial fermentation. Despite a wide variation in starch digestion kinetics between starch ingredients commonly used in pig feed [5], digested starch is considered to have a fixed nutritional value regardless its source. This value is based on its energy supply for maintenance and productive processes, such as growth; the so-called net energy value. The net energy value of digested starch is greater than that of fermented starch, i.e., resistant starch (RS), which is generally assumed to be fermented in a similar manner as dietary fibers or non-starch polysaccharides, consequently yielding about 30% less net energy [6]. A more accurate prediction of the energetic value of both digested and fermented starch may improve future feed formulation and as a result raw material utilization and feed efficiency in swine, consequently contributing to a more efficient use of resources.

1.2 Starch digestibility and metabolism in pigs

Starch is a polysaccharide composed of amylose and amylopectin, which is mainly digested by pancreatic α-amylase in the small intestine of pigs. The contribution of salivary α-amylase to starch digestion is considered to be small, as was shown in humans [7]. This is explained by a short retention time of feed in the mouth and rapid inactivation of salivary α-amylase in de acidic environment of the stomach. In pigs, breakdown of starch in the stomach is limited as well and predominantly explained by microbial fermentation [8, 9]. Final end-products of starch digestion by α-amylase are mainly maltose and maltotriose. However, α-amylase cannot hydrolyze 1,6-linkages, which are mainly present in amylopectin and to a small extent (<1%) in amylose [10], and is also not able to break down the α-1,4-linkage adjacent to this branching point. Therefore a great part of the final end products of amylopectin digestion by α-amylase exist of α–limit dextrins [11, 12]. All end-products of starch hydrolysis by α-amylase are subsequently hydrolyzed to glucose by brush border enzymes glucoamylase, sucrase-isomaltase, and α-dextrinase (Figure 1.1). Glucoamylase is

initially the most effective glycosidase to remove single glucose units from the non-reducing end of the α-1,4-chain, α-dextrinase is the only enzyme capable to break down α-1,6-linkages, and sucrase-isomaltase is the preferred glucosidase for removing the final glucose units after the chain is been reduced to two or three units [11].
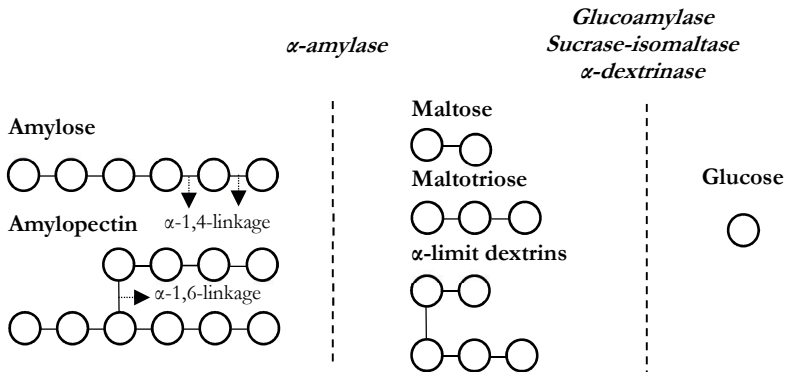

Figure 1.1 Enzymatic hydrolysis of amylose and amylopectin into glucose. Each circle represents a glucose molecule.

1.2.1 Absorption and post-absorptive use of glucose

Absorption of free glucose from the gut is regulated by a sodium-dependent active process through the transport protein SGLT1. The sodium pump creates a sodium gradient across the membrane, by using sodium/potassium ATPase, facilitating glucose to cross the membrane of the enterocyte [13]. Subsequently, glucose is transported from the enterocyte into the hepatic portal vein through glucose transporter GLUT2. Glucose uptake from the blood by body tissues is carried out by carrier-mediated diffusion and metabolic trapping through phosphorylation of glucose into glucose-6-phosphate by either hexokinase or glucokinase [14]. Hexokinase is present in all mammalian body tissues, whereas glucokinase is only present in the liver, pancreas, gut and brain. Hexokinase has a much higher affinity for glucose than glucokinase. Therefore, hexokinase will act under all conditions while glucokinase is only active during the fed state when the glucose concentration in the portal vein is significantly increased [14]. Main transporters facilitating the uptake of glucose from the blood by body tissues are GLUT1, GLUT2, GLUT3, and GLUT4. GLUT1 and GLUT3 are particularly expressed in the brain and responsible for glucose uptake in the fasting state. GLUT2 is expressed in kidneys, pancreatic β-cells, and liver. In pancreatic β-cells GLUT2 is thought to be responsible for glucose-sensing mechanisms while in the liver GLUT2 allows for bi-directional transport of glucose [15]. GLUT4 is mainly expressed in heart, adipose- and muscle tissues. In contrast to the other glucose transporters, GLUT4 is insulin sensitive, and is most active when postprandial glucose levels are high [16]. Intracellularly,

glucose is phosphorylated to glucose-6-phosphate, which enters the glycogenesis to be stored as glycogen or enters the glycolysis to be oxidized, reduced to lactate or stored as fat through *de novo* fatty acid synthesis.

1.2.2 Regulation of glucose metabolism

The metabolic hormone insulin is responsible for clearance of rising postprandial glucose levels, by facilitating glucose uptake through GLUT4. To stimulate glucose uptake by muscle and adipose tissues, insulin stimulates glycogen synthesis in liver and muscle tissues, protein synthesis in muscle tissues, and *de novo* lipogenesis in adipose tissues. At the same time insulin inhibits *de novo* gluconeogenesis and glucose release in the liver by suppressing the secretion of glucagon [17, 18]. In the fasted state, glucagon stimulates glycogenolysis in the liver and muscles, lipolysis in adipose and muscle tissues and liver, proteolysis in muscle tissues, and *de novo* gluconeogenesis in the liver [19]. Initial insulin release is triggered by stimulation of the vagus nerve [20, 21] in anticipation to the meal during the cephalic phase (sight, smell, and taste of food) [22, 23], by stimulation of the release of incretins GLP-1 and GIP by responding epithelial cells to nutrients in the gut [24], and by intake of essential amino acids leucine, lysine, and arginine [25]. In humans, the incretin effect accounts for approximately 50 to 70 per cent of the total insulin release [24]. After absorption of glucose from the gut, insulin release continues as a response to elevated postprandial blood glucose levels. When finally the postprandial blood glucose concentration drops, insulin secretion is down-regulated and remaining insulin in the blood is broken-down by the liver and kidneys [26]. Subsequently, glucagon release is no longer suppressed by insulin inhibiting the uptake of glucose by body cells. Because only GLUT2 allows for bi-directional transport [15], the liver is particularly responsible for maintaining basal glucose levels in the fasting state.

1.3 Metabolic fate of undigested starch

Carbohydrates that resist digestion, like RS, serve as a substrate for microbial fermentation. Microbial fermentation occurs in all compartments of the gastro-intestinal tract; however, the main site for microbial fermentation in pigs is the hindgut [27]. First, undigested carbohydrates are hydrolyzed into monosaccharides, which are subsequently fermented through the glycolytic pathway (six-carbon sugars) or pentose-phosphate pathway (five-carbon sugars) generating ATP [28]. During this process organic end-products are formed, which serve as hydrogen acceptors that are subsequently secreted as waste metabolites. The major metabolites produced are short-chain fatty acids (SCFA) acetate, propionate, and butyrate, and gasses carbon dioxide, hydrogen and methane. Depending on the bacteria degrading the substrate, lactate, ethanol and succinate may be produced as well [29]. Short-chain fatty acids are rapidly absorbed and subsequently utilized by the host; when infused

in the caecum less than 1% of the infused SCFAs was recovered in the feces of pigs [30]. The majority of absorbed butyrate is utilized for energy in the epithelial cells of the colon [31-33]. Residual butyrate and propionate are taken up by the liver [34], where butyrate is used for generating energy and propionate is mainly used for gluconeogenesis [35]. Acetate is less easily taken up by the liver (70%) where it is oxidized or used to synthesize cholesterol and long-chain fatty acids. Its remainder is metabolized by other body tissues, including heart, kidney, adipose, and muscle tissues [36].

1.4 The rate and extent of starch digestion

The rate of starch digestion affects the site of degradation in the small intestine, whereas the extent determines the amount of undigested starch that is available for microbial fermentation. *In vitro*, the rate and extent of starch digestion are determined by incubation of starch with pancreatic amylase and amyloglucosidase [37]. In this method, starch is classified into three categories; rapidly digestible (RDS), slowly digestible (SDS), and resistant starch (RS), based on the glucose release from 0 - 20 minutes, 20 - 120 minutes, and after 120 minutes, respectively. For unprocessed feed ingredients used in pig feed, the RDS, SDS, and RS fractions varies between 9 – 40 %, 15 – 60 %, and 5 – 76 %, respectively [5, 38-40]. Resistant starch can be classified into physically inaccessible starch, such as partly milled grains and seeds (RS1), resistant starch granules, present in high amylose starch (RS2), retrograded starch (RS3), and chemically modified starch (RS4). *In vivo,* starch digestibility is often measured as starch disappearance from the intestinal lumen, and consequently does not distinguish digested from fermented starch. Because it is assumed that fermentation in the upper gastrointestinal tract is negligible, ileal starch disappearance often represents the maximum hydrolysis of starch by host-enzymes. In addition, *in vivo* starch digestion kinetics can be measured indirectly using net portal glucose concentrations [41]. Total postprandial net portal glucose appearance reflects the maximum degradation of starch (area under the curve; Figure 1.2), whereas the time that net portal glucose concentrations require to peak reflects the composite effect of passage rate and the starch digestion rate. Net portal glucose appearance, however, is only an approximation of starch digestion as part of the absorbed glucose may be utilized by the intestine or fermented by microbes [41-44]. In humans, the glycemic index is used to create a relative ranking of digestible rates of carbohydrates in foods according to how much they increase postprandial blood glucose levels [45]. Rapidly digestible starch sources have a glycemic index near one; starch is more or less assumed to be completely hydrolyzed in the small intestine. Slowly digestible starch sources have a glycemic index significantly below one; a proportion of the starch remains undigested and serves as a substrate for microbial fermentation. Starch resistant to enzymatic hydrolysis has a glycemic index of zero; all starch is fermented by microbes.
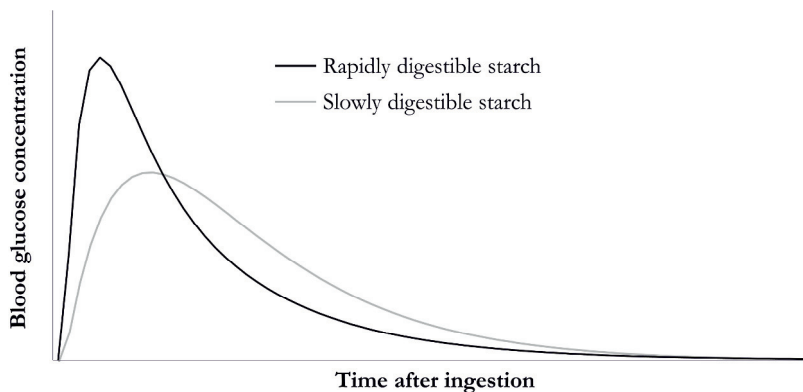
Figure 1.2 Response in postprandial blood glucose concentrations after ingestion of rapidly and slowly digestible starch.

1.4.1 Factors affecting starch digestion kinetics and glycemic response

During seed formation in plants, starch is deposited in granules located in the endosperm. The granules are composed of layers with varying amylose and amylopectin contents. An increase in the ratio between amylose and amylopectin inside the starch granule is negatively correlated with the rate of starch digestion [46]. This relation is explained by a reduced surface area and more intra-molecular hydrogen bonds for amylose than amylopectin. Although a higher amylose content reduces the rate of starch digestion, the crystalline region consisting of short-branch chains of amylopectin may decrease enzyme accessibility decreasing the rate of starch digestion. Recently, Martens*, et al.* [47] concluded that variation in the *in vitro* rate of starch digestion between botanic sources is mainly explained by the type of crystalline structure and by its amylopectin chain length distribution. Another factor that is suggested to affect starch digestibility is granule size. Small starch granules, with a relatively large surface area available for enzyme binding, are more easily digested by enzymes compared to large starch granules [48]. In addition, interactions with surface compounds reduce surface area by blocking the access of adsorption sites for enzymes [49]. The two most important interactions with starch are complexes formed between fatty acids and amylose [50, 51], and the interaction of starch with the protein matrix surrounding the starch granule [52]. A high density of the protein matrix itself may lower accessibility for enzymes as well. According to Lynn and Cochrane [53], initial digestion of starch occurs in the interior of the starch granule suggesting that the dependency of surface area on starch digestion rate is less important than assumed. Presence of pores and pits in the starch granule surface facilitates the entry of α-amylase stimulating its digestion. Furthermore, besides its native form, starch properties affecting starch digestibility are also affected by feed processing. During cooking starch gelatinizes and loses its crystallinity which makes it

more accessible for enzymes. However, cooled gelatinized starch may partly recrystallize; so-called retrograded starch is poorly digested [37].

Besides its intrinsic properties, starch digestion and its glycemic response are also affected by extrinsic factors such as the concentration of pancreatic amylase, presence of and interaction with other nutrients in the feed matrix, and transit time from mouth to terminal ileum [37, 54]. These processes involved in the digestion of starch may be influenced by presence of anti-nutritional factors, soluble fibers, and certain organic acids [55-57]. Some raw materials used in pig feed, such as rye, wheat, and oats contain α-amylase inhibitors in varying concentrations. For example, α-amylase inhibitors present in wheat inhibit mammalian salivary amylase activity [58]. In addition, phytic acid may interact with amylase proteins or binds to salivary minerals such as calcium which is known to catalyze amylase activity [59]. In addition, viscous dietary fibers may reduce the rate of gastric emptying and motility of chyme in the small intestine [60] affecting the exposure time of starch to enzymatic digestion and consequently its glycemic response.

1.5 Consequences for pig performance

A slow starch digestion is suggested to have positive effects on growth performance of pigs [61]. This is explained by the attenuated response in postprandial insulin release with slowly digestible starch when compared to rapidly digestible starch. Insulin triggers the uptake of glucose by insulin-responsive muscle and adipose tissues increasing protein and fat synthesis. At the same time, the release of glucose from glycogen and non-esterified fatty acids from adipose tissues is suppressed. Shifts in these metabolic processes as a response to fluctuations in blood insulin levels are probably less explicit with slowly than with rapidly digestible starch, which is suggested to have a positive effect on animal performance [69]. Apart from the rate, the extent of starch digestion may affect pig performance as well. Because fermented carbohydrates yield less energy than digested carbohydrates [6], a low extent of ileal starch digestion is assumed to compromise animal performance [6, 62, 63]. The lower energy value of fermented starch than digested starch is explained by greater energy losses through microbial fermentation, and a lower efficiency to utilize SCFAs than glucose for energy [62]. The effect of starch digestion and fermentation on energy utilization and consequently pig performance is illustrated in figure 1.3.
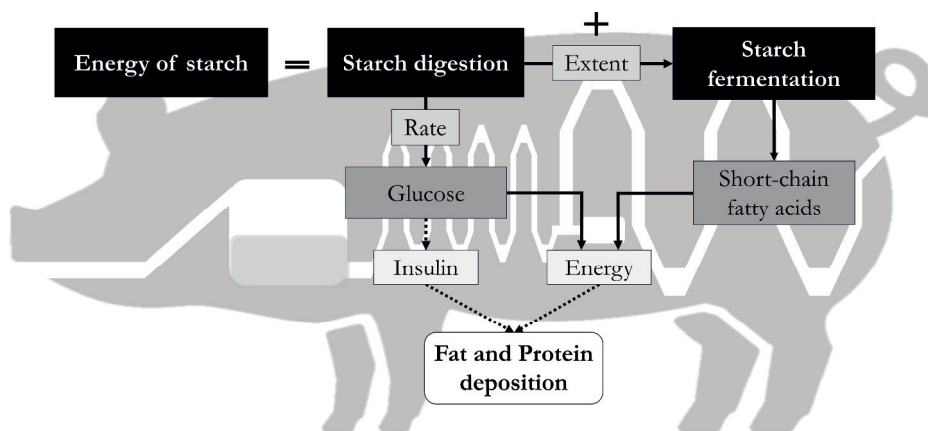
Figure 1.3 Effect of starch digestion kinetics on energy utilization, and consequently protein and fat deposition in pigs. The energy value of starch equals the sum of energy from digested and fermented starch. The extent of digestion determines the size of these proportions, and consequently the amount of glucose and short-chain fatty acids that both can be used as energy source by the pig. The rate of starch digestion affects postprandial insulin response, which subsequently stimulates protein and fat deposition.

1.5.1 State of the art: starch digestion kinetics and pig performance

Literature on the effect of starch digestion kinetics on pig performance is inconclusive. Feeding slowly digestible starch may increase feed intake, growth rate and efficiency, and stimulate leanness in pigs [39, 61, 64], which was explained by a reduced stimulation of satiety and fat accretion by insulin and glucose. Others, however, reported no positive effect of slowly digestible starch on growth or feed efficiency in restrictedly-fed pigs [65], or reported a positive correlation between the rate of starch digestion and protein deposition [66], indicating that not slowly but rapidly digestible starch stimulates faster growth and leanness. According to the assumption that fermented starch yields less energy than digested starch, growth rates of individually housed restricted-fed pigs fed high-RS diets were compromised by a lower energy intake [65, 67]. Under *ad libitum* conditions, however, growth rates and efficiencies of pigs fed high-RS diets were either unaffected [39, 68] or improved compared to pigs fed low-RS diets [61]. Fouhse and Zijlstra [69] suggested that the physiological response corresponding to RS intake is affected by feeding regime, which may explain the difference in findings. Under unrestricted feeding conditions, feed is gradually consumed during the day resulting in consumption of smaller and more frequent meals compared to restricted feeding. Because the magnitude of the postprandial increase of blood insulin levels decreases with feeding frequency, as was shown in veal calves [70] and humans [71], effects of starch digestion kinetics on postprandial insulin levels are possibly more pronounced under restricted than under *ad libitum* conditions.

1.5.2 Possible health benefits of slowly digestible and resistant starch

Many studies with humans suggest that a slower rate of starch digestion has a beneficial effect on common chronic diseases, like diabetes, insulin resistance, cardiovascular diseases, and obesity (reviewed by Miao, *et al.* [72]), which is explained by a slower and prolonged glycemic response with slowly digestible starch than with rapidly digestible starch [72]. Metabolic disorders may also exist in pigs. Paredes, *et al.* [73] showed that poor performing pigs have a reduced insulin sensitivity compared with high performing pigs at 6 weeks of age. As a result of reduced insulin sensitivity hyperinsulinemia may occur. In human children of normal birth weight, reduced insulin sensitivity is associated with hypoxia during the birth process [74]. Although literature describing this phenomenon in piglets is not available, hypoxia in piglets is shown to be related to a decrease in growth, colostrum intake, and prolongation of the first udder contact [75]. Slowly digestible starch reduces the postprandial insulin response, and therefore may help low performance pigs to cope with postprandial increases in blood glucose [73]. In addition, RS is suggested to be beneficial for maintenance of gut health and reduction of risk factors for intestinal problems [76], as it stimulates microbial production of butyrate [77, 78]. Butyrate is suggested to stimulate gut epithelium growth, cell differentiation, and immune system [76], and may act as a prebiotic by promoting population of commensal gut microbes in pigs [65, 79, 80].

1.6 Voluntary feed intake of pigs

When feed is available *ad libitum*, feeding by pigs occurs in successive visits with intermittent breaks, which can be clustered into meals [81, 82]. The size of each meal is controlled by short-term satiety signals that are triggered by, amongst other mechanisms, gastric distention [83] and the "ileal break" mechanism. The "ileal break" mechanism reduces gastric emptying and passage rate of nutrients in the ileum in order to optimize nutrient digestion [84, 85]. The onset of a meal is stimulated by hunger, which is triggered by a decrease in satiety; the feeling of fullness that persists after eating and suppresses further consumption [86]. The size of a meal is determined by satiation; the process that leads to the termination of a meal [86]. Because under *ad libitum* conditions hunger may not persist, Maselyne, *et al.* [87] suggested that voluntary feed intake is controlled by meal size rather than the interval between meals. Voluntary feed intake, however, may vary between pigs due to intrinsic differences affected by body weight [88, 89], gender [90], genotype [90-92], or health status [93, 94], and is affected by environmental factors, such as dietary composition, housing conditions, and behavioral cues, such as the light/dark schedule [85]. For example, group housed pigs have less, but larger meals, and eat faster than individual housed pigs [95]. In addition, low ambient temperatures (cold stress) increases feed intake of growing pigs [96, 97], within their physical capacity [94], to compensate for increased energy losses, whereas

high ambient temperatures (heat stress) decreases feed intake [89, 98] possibly to decrease postprandial heat production.

1.6.1 Timing of feed intake

The timing of food intake is triggered by energy-deficiency signals, and simultaneously constrained by the endogenous circadian clock [99]. The body's central clock is set by the superchiasmatic nucleus of the hypothalamus [100, 101], which generates circadian rhythms in behavioral and physiological processes, such as rest and activity, core body temperature, neuroendocrine function, autonomic function, and memory and psychomotor performance [100]. The central clock is entrained by periodic environmental cues called zeitgebers, e.g., light-dark (L/D) cycle, feed availability, and ambient temperature [102]. When feed is available *ad libitum* and pigs are exposed to 8 h of darkness and 16 h of light, the circadian feeding pattern of pigs is characterized by two peaks: a small peak in the morning, and a large peak in the afternoon [81, 103, 104] (Figure 1.4).
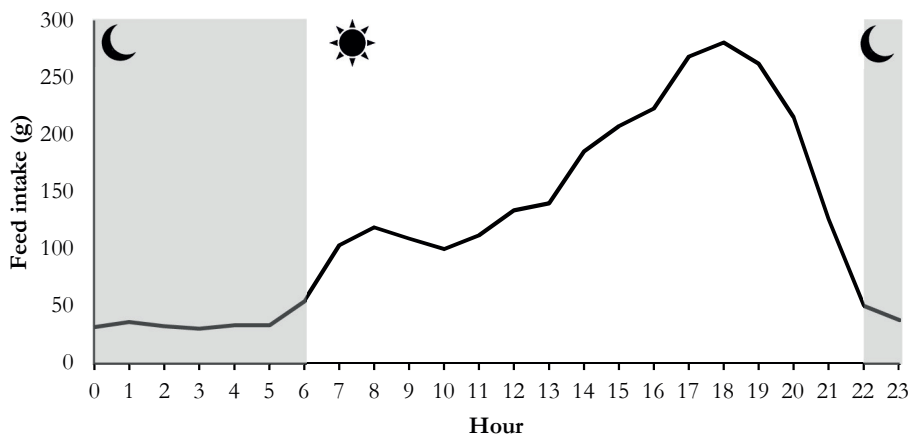
Figure 1.4 Circadian feeding pattern of growing pigs (70-115 kg; n=279) under *ad libitum* conditions (Van Erp *et al.*, unpublished data). The shaded area represents the period in where the lights were switched off.

Boumans*, et al.* [105] showed that this feeding pattern can be associated with the circadian rhythmicity in cortisol and melatonin levels; shortly after light onset melatonin level drops while feed intake increases, whereas before the onset of darkness cortisol level increases together with intake of feed. Circadian rhythms in cortisol and melatonin levels are entrained by the L/D cycle and are independent of feeding [106, 107]. Consequently, when exposed to 24 hours of light, feeding patterns of pigs housed under *ad libitum* feeding conditions change; feed intake peaks in the morning and decreases gradually during the day [108]. These results confirm that feeding patterns of pigs are entrained by the L/D cycle, hence are

orchestrated by the endogenous central clock. However, when pigs are group housed, increased aggression and competition may change endogenous feeding rhythms of pigs as weaker pigs may be chased away from the feeder by more dominant pigs [103]. Consequently, the submissive pig tries to maintain its feeding level by postponing feeding to less desirable periods of the day. Under more extreme conditions, with highly stocked pens, a lack of feeding space or insufficient feeding time may even force pigs to eat during the night [109]. In addition, heat stress forces pigs to shift their feed intake from day to night [89, 110], indicating that feed intake, in addition to the L/D cycle, may be affected by circadian variation in ambient temperatures as well. Forced changes in meal patterns of pigs may affect their performance and health. In humans, misalignment between natural food intake rhythms and the circadian clock due to e.g., shift work, (social) jet lag, or night eating syndrome is associated with a greater risk for metabolic disorders like obesity [111-114], reduced insulin resistance [115], diabetes type 2 [116, 117], or cardiometabolic diseases [118, 119].

1.6.2 Effect of starch on satiety regulation and meal patterns of pigs

Feed intake of pigs is controlled by satiety regulating mechanisms that are responding to, amongst other nutrients, glucose appearance in the oral cavity [23] and gut [120], hence the rate and extent of starch digestion. The appearance of glucose stimulates satiety directly through stimulation of the vagus nerve [121] or through stimulation of K cells secreting incretin hormone glucagon-like peptide 1 (GLP-1). GLP-1 plays an important role in feed intake control as it stimulates the release of insulin [121-123]. In addition, GLP-1 is also suggested to suppress appetite directly as it mediates the "ileal brake" mechanism [84]. In contrast to digestible starch, RS stimulates microbial production of SCFAs in the large intestine, which slows down gastric emptying [124], possibly by stimulating peptide tyrosine-tyrosine (PYY) release [125]. In addition, in rats, SCFAs have been shown to stimulate secretion of GLP-1 [126, 127]. Because satiety regulation is responding differently to intake of digestible and resistant starch, feed intake patterns may change accordingly when feed is available *ad libitum*; Da Silva*, et al.* [68] showed that pigs fed high-RS diets have less, but larger meals per day compared with pigs fed high digestible starch diets. It is suggested in both humans [128, 129] and pigs [68] that production of SCFAs in pigs fed high-RS diets prolongs the energy supply to the body extending satiety, and thus postponing the feeling of hunger, whereas a greater insulin response in pigs fed low RS diets stimulates satiation. A simplified model of the effect of digested and fermented starch on satiety regulation, and consequently meal patterns of pigs is illustrated in figure 1.5. In addition, pigs eat more to maintain daily energy intake when fed a diet with a lower energy content [130], e.g., RS-diets, which, as earlier explained, are assumed to have a lower energy content than digestible starch diets [6].
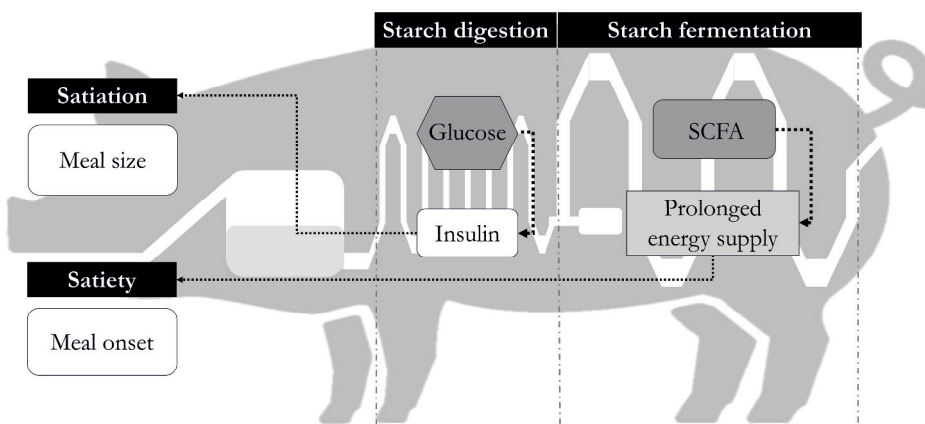
Figure 1.5 Simplified model of effects of digested and fermented starch on meal patterns of pigs. Starch is digested into glucose, which gut and portal vein appearance stimulates the release of insulin. Microbial fermentation of undigested starch results in production of short-chain fatty acids (SCFA). Rising postprandial blood glucose and insulin levels stimulate satiation, thereby initiating the ending of a meal determining meal size, whereas a gradual and continuous absorption of SCFAs throughout the day may serve as a prolonged energy supply stimulating satiety delaying meal onset.

1.7 Thesis aim and outline

There are indications that both the rate and extent of starch digestion influence pig performance. But to date, evidence in literature on the effect of starch digestion kinetics on pig performance is not conclusive. Some studies have shown that slowly digestible starch increases growth efficiency and leanness of pigs [39, 61, 64], whereas others reported no or opposite effects [65, 66]. Moreover, it is commonly assumed that RS, like other indigestible carbohydrates, delivers less energy than digestible starch. Despite its lower energy value, however, growth rates of pigs fed RS were similar to [39, 68] or greater than [61] those of pigs fed digestible starch when feed is available *ad libitum*. This thesis aims to improve understanding of underlying processes affecting the utilization of digested and fermented starch, such as digestive processes and meal patterns, to ultimately predict the effect of starch digestion kinetics on pig performance more accurately. The outline of the research chapters of this thesis is illustrated in figure 1.6.

Alterations in meal patterns may interact with the decrease in postprandial glucose and insulin responses when RS is replaced for digestible starch [41, 65], affecting its utilization and ultimately growth performance of pigs. Therefore, the effect of RS on digestive processes, in combination with its effect on meal patterns and pig performance under *ad libitum* feeding conditions is investigated in chapter 2.

A slower rate of starch digestion is suggested to reduce fat deposition of growing pigs [61] and to be beneficial for growth retarded pigs [73] through its decreasing effect on postprandial insulin levels. In chapter 3, the effect of the rate of starch digestion on energy efficiency is investigated in slow- and fast growing pigs; two populations of pigs that previously were demonstrated to differ in insulin sensitivity [73]. Because slowly digestible starch reduces postprandial insulin release, it was hypothesized that it may alleviate the consequences of a reduced insulin sensitivity for growth in slow growing pigs.

Currently, the proportion of starch fermented in the small intestine is often neglected. Ileal starch disappearance is assumed to represent the maximum hydrolysis of starch by host-enzymes, and consequently determines the proportion of starch digested and fermented affecting its energy value. In chapter 4, we aim to quantify the contribution of ileal starch fermentation to ileal starch disappearance to get a better indication of total starch digested.

Alterations in meal patterns to changes in RS intake may be dynamic, depending on the adaptation of processes involved. To gain understanding of the mode of action on the effects of RS on meal patterns, we investigated short-term changes in meal patterns of growing pigs as a response to dietary RS and digested starch intake in chapter 5.

Under *ad libitum* conditions, feed intake patterns of pigs follow a clear circadian rhythm [81]. In chapter 6 of this thesis, we investigate if deviating from this pattern, when forced to eat during the night, affects energy metabolism in pigs.

Finally, in chapter 7 findings of all research chapters are combined and discussed in relation to the overall research aim of this thesis, and recommendations for the pig industry and future research are given.
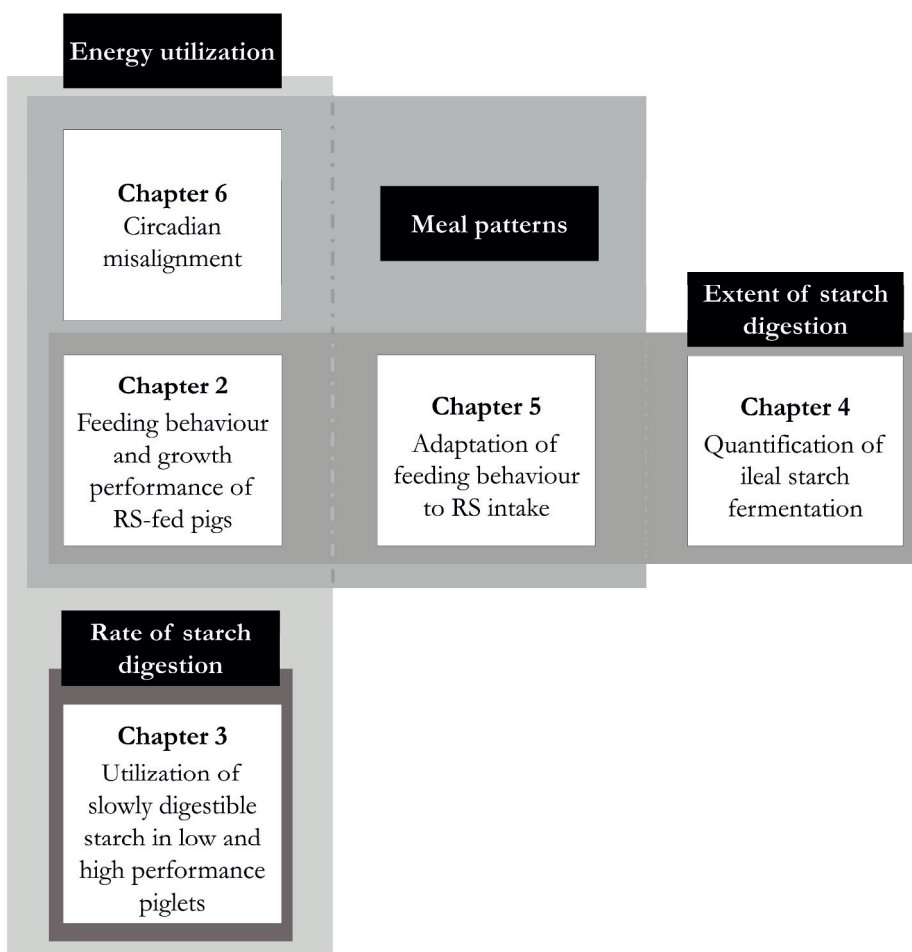
Figure 1.6 Outline of thesis. Chapters 2 to 6 are clustered (rectangles) into 4 topics; energy utilization (2,3,6), meal patterns (2,5,6), extent of starch digestion (2,4,5), and rate of starch digestion (3).

References

1.   FAO: World food and agriculture: Statistical pocketbook Rome: Food and Agricultural Organization of the United Nations; 2018.

2.   FAO: How to Feed the World in 2050. Rome: Food an Agricultural Organization of the United Nations; 2009.

3.   FAO: Save and grow in practice: maize rice wheat. Rome: Food and Agricultural Organization of the United Nations; 2016.

4.   Knudsen KEB, Lærke HN, Steenfeldt S, Hedemann MS, Jørgensen H: In vivo methods to study the digestion of starch in pigs and poultry. *Anim Feed Sci Tech* 2006, 130:114-135.

5.   Giuberti G, Gallo A, Cerioli C, Masoero F: *In vitro* starch digestion and predicted glycemic index of cereal grains commonly utilized in pig nutrition. *Anim Feed Sci Tech* 2012, 174:163-173.

6.   Blok MC, Brandsma G, Bosch G, Gerrits WJJ, Jansman AJM, Fledderus J, Everts H: A new Dutch net energy formula for feed and feedstuffs for growing and fattening pigs. In *CVB-Documentation Report*. Wageningen, the Netherlands: Centraal Veevoeder Bureau; 2015.

7.   Fried M, Abramson S, Meyer J: Passage of salivary amylase through the stomach in humans. *Dig Dis Sci* 1987, 32:1097-1103.

8.   Drochner W: Digestion of carbohydrates in the pig. *Archives of animal nutrition* 1993, 43:95-116.

9.   Martens BM, Bruininx E, Gerrits WJJ, Schols H: The importance of gastric amylases in starch digestion kinetics in pigs. In *Starch digestion kinetics in pigs: The impact of starch structure, feed processing, and digesta passage behaviour*. Edited by Martens BM. Wageningen2019

10.   Joye IJ: Starch. In *Encyclopedia of food chemistry. Volume* 1. Edited by Varelis P, Melton L, Shahidi F. Cambridge, US: Elsevier; 2019

11.   Gray GM: Starch digestion and absorption in nonruminants. *J Nutr* 1992, 122:172.

12.   Robyt J: Enzymes and their action on starch. . In *Starch: Chemistry and Technology. Volume* 237-292. Edited by BeMiller J, Whistler R: Academic Press; 2009

13.   Stevens BR, Kaunitz JD, Wright EM: Intestinal transport of amino acids and sugars: advances using membrane vesicles. *Annu Rev Physiol* 1984, 46:417-433.

14.   Ballard F: Glucose utilization in mammalian liver. *Comp Biochem Physiol* 1965, 14:437-443.

15.   Leturque A, Brot-Laroche E, Le Gall M, Stolarczyk E, Tobin V: The role of GLUT2 in dietary sugar handling. *J Physiol Biochem* 2005, 61:529.

16.   Wood IS, Trayhurn P: Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. *Br J Nutr* 2003, 89:3-9.

17.   Khan A, Pessin J: Insulin regulation of glucose uptake: a complex interplay of intracellular signalling pathways. *Diabetologia* 2002, 45:1475-1483.

18.   Cross DA, Watt PW, Shaw M, Van der Kaay J, Downes CP, Holder JC, Cohen P: Insulin activates protein kinaseB, inhibits glycogen synthase kinase-3 and activates glycogen synthase by rapamycin-insensitive pathways in skeletal muscle and adipose tissue. *FEBS Lett* 1997, 406:211-215.

19.   Jiang G, Zhang BB: Glucagon and regulation of glucose metabolism. *Am J Physiol Endocrinol Metab* 2003, 284:E671-E678.

20.   Holst JJ, Gronholt R, Schaffalitzky de Muckadell OB, Fahrenkrug J: Nervous control of pancreatic endocrine secretion in pigs. I. Insulin and glucagon responses to electrical stimulation of the vagus nerves. *Acta Physiol Scand* 1981, 111:1-7.

21. Marty N, Dallaporta M, Thorens B: Brain glucose sensing, counterregulation, and energy homeostasis. *Physiology* 2007, 22:241-251.
22. Teff K: Nutritional implications of the cephalic-phase reflexes: endocrine responses. *Appetite* 2000, 34:206-213.
23. Thorens B: Brain glucose sensing and neural regulation of insulin and glucagon secretion. *Diabetes Obes Metab* 2011, 13:82-88.
24. Gautier J-F, Choukem S-P, Girard J: Physiology of incretins (GIP and GLP-1) and abnormalities in type 2 diabetes. *Diabetes Metab* 2008, 34:S65-S72.
25. Floyd Jr JC, Fajans SS, Conn JW, Knopf RF, Rull J: Stimulation of insulin secretion by amino acids. *J Clin Invest* 1966, 45:1487.
26. Duckworth WC, Bennett RG, Hamel FG: Insulin degradation: Progress and Potential. *Endocr Rev* 1998, 19:608-624.
27. Jensen BB, Jørgensen H: Effect of dietary fiber on microbial activity and microbial gas production in various regions of the gastrointestinal tract of pigs. *Appl Environ Microbiol* 1994, 60:1897-1904.
28. Miller TL, Wolin MJ: Pathways of acetate, propionate, and butyrate formation by the human fecal microbial flora. *Appl Environ Microbiol* 1996, 62:1589-1592.
29. Drochner W, Kerler A, Zacharias B: Pectin in pig nutrition, a comparative review. *J Anim Physiol Anim Nutr (Berl)* 2004, 88:367-380.
30. Jørgensen H, Larsen T, Zhao X-Q, Eggum BO: The energy value of short-chain fatty acids infused into the caecum of pigs. *Br J Nutr* 1997, 77:745-756.
31. Roediger W: Utilization of nutrients by isolated epithelial cells of the rat colon. *Gastroenterology* 1982, 83:424-429.
32. den Besten G, Van Eunen K, Groen AK, Venema K, Reijngoud D-J, Bakker BM: The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res* 2013, 54:2325-2340.
33. Roediger WE: The place of short-chain fatty acids in colonocyte metabolism in health and ulcerative colitis: the impaired colonocyte barrier. *Physiological and Clinical Aspects of Short-chain fatty acids* 1995, 337351.
34. Bloemen JG, Damink SWO, Venema K, Buurman WA, Jalan R, Dejong CH: Short chain fatty acids exchange: Is the cirrhotic, dysfunctional liver still able to clear them? *Clin Nutr* 2010, 29:365-369.
35. Roy CC, Kien CL, Bouthillier L, Levy E: Short-chain fatty acids: ready for prime time? *Nutr Clin Pract* 2006, 21:351-366.
36. Knowles SE, Jarrett IG, Filsell OH, Ballard FJ: Production and utilization of acetate in mammals. *Biochem J* 1974, 142:401-411.
37. Englyst HN, Kingman S, Cummings J: Classification and measurement of nutritionally important starch fractions. *Eur J Clin Nutr* 1992, 46:S33-50.
38. Zhang G, Ao Z, Hamaker BR: Slow digestion property of native cereal starches. *Biomacromolecules* 2006, 7:3252-3258.
39. Doti S, Suárez-Belloch J, Latorre M, Guada J, Fondevila M: Effect of dietary starch source on growth performances, digestibility and quality traits of growing pigs. *Livest Sci* 2014, 164:119-127.
40. Sun T, Lærke HN, Jørgensen H, Knudsen KEB: The effect of extrusion cooking of different starch sources on the *in vitro* and in vivo digestibility in growing pigs. *Anim Feed Sci Tech* 2006, 131:67-86.

41. Van Kempen TA, Regmi PR, Matte JJ, Zijlstra RT: *In vitro* starch digestion kinetics, corrected for estimated gastric emptying, predict portal glucose appearance in pigs. *J Nutr* 2010, 140:1227-1233.

42. Van der Meulen J, Bakker G, Bakker J, De Visser H, Jongbloed A, Everts H: Effect of resistant starch on net portal-drained viscera flux of glucose, volatile fatty acids, urea, and ammonia in growing pigs. *J Anim Sci* 1997, 75:2697-2704.

43. Deutz N, Ten Have G, Soeters P, Moughan P: Increased intestinal amino-acid retention from the addition of carbohydrates to a meal. *Clin Nutr* 1995, 14:354-364.

44. Vangsøe CT, Ingerslev AK, Theil PK, Hedemann MS, Lærke HN, Knudsen KEB: *In vitro* starch digestion kinetics of diets varying in resistant starch and arabinoxylan compared with in vivo portal appearance of glucose in pigs. *Food Research International* 2016, 88:199-206.

45. Jenkins D, Wolever T, Taylor RH, Barker H, Fielden H, Baldwin JM, Bowling AC, Newman HC, Jenkins AL, Goff DV: Glycemic index of foods: a physiological basis for carbohydrate exchange. *Am J Clin Nutr* 1981, 34:362-366.

46. Svihus B, Uhlen AK, Harstad OM: Effect of starch granule structure, associated components and processing on nutritive value of cereal starch: A review. *Anim Feed Sci Tech* 2005, 122:303-320.

47. Martens BM, Gerrits WJ, Bruininx EM, Schols HA: Amylopectin structure and crystallinity explains variation in digestion kinetics of starches across botanic sources in an *in vitro* pig model. *J Anim Sci Biotechno* 2018, 9:91.

48. Tester R, Qi X, Karkalas J: Hydrolysis of native starches with amylases. *Anim Feed Sci Tech* 2006, 130:39-54.

49. Oates CG: Towards an understanding of starch granule structure and hydrolysis. *Trends Food Sci Technol* 1997, 8:375-382.

50. Godet M, Bizot H, Buléon A: Crystallization of amylose—fatty acid complexes prepared with different amylose chain lengths. *Carbohydr Polym* 1995, 27:47-52.

51. Cui R, Oates C: The effect of retrogradation on enzyme susceptibility of sago starch. *Carbohydr Polym* 1997, 32:65-72.

52. Brennan C, Amor M, Harris N, Smith D, Cantrell I, Griggs D, Shewry P: Cultivar differences in modification patterns of protein and carbohydrate reserves during malting of barley. *J Cereal Sci* 1997, 26:83-93.

53. Lynn A, Cochrane MP: An evaluation of confocal microscopy for the study of starch granule enzymatic digestion. *Starch-Stärke* 1997, 49:106-110.

54. Chapman RW, Sillery JK, Graham MM, Saunders DR: Absorption of starch by healthy ileostomates: effect of transit time and of carbohydrate load. *Am J Clin Nutr* 1985, 41:1244-1248.

55. Biliaderis CG: The structure and interactions of starch with food constituents. *Can J Physiol Pharmacol* 1991, 69:60-78.

56. Björck I, Liljeberg H, Östman E: Low glycaemic-index foods. *Br J Nutr* 2000, 83:S149-S155.

57. Pi-Sunyer FX: Glycemic index and disease. *Am J Clin Nutr* 2002, 76:290S-298S.

58. Lankisch M, Layer P, Rizza RA, DiMagno EP: Acute postprandial gastrointestinal and metabolic effects of wheat amylase inhibitor (WAI) in normal, obese, and diabetic humans. *Pancreas* 1998, 17:176-181.

59. Singh J, Dartois A, Kaur L: Starch digestibility in food matrix: a review. *Trends Food Sci Technol* 2010, 21:168-180.

60. Braaten JT, Wood PJ, Scott FW, Riedel KD, Poste LM, Collins MW: Oat gum lowers glucose and insulin after an oral glucose load. *Am J Clin Nutr* 1991, 53:1425-1430.

61.   Van Kempen T, Pujol S, Tibble S, Balfagon A: *In vitro* characterization of starch digestion and its implications for pigs. In *Paradigms in Pig Science*. Edited by Wiseman J, Varley M, Mcorist S, Kemp B. Nottingham: Nottingham University Press; 2007: 515-525

62.   Noblet J, Le Goff G: Effect of dietary fibre on the energy value of feeds for pigs. *Anim Feed Sci Tech* 2001, 90:35-52.

63.   Gerrits WJ, Bosch MW, Van den Borne JJ: Quantifying resistant starch using novel, in vivo methodology and the energetic utilization of fermented starch in pigs. *J Nutr* 2012, 142:238-244.

64.   Li T, Huang R, Wu G, Lin Y, Jiang Z, Kong X, Chu W, Zhang Y, Kang P, Hou Z: Growth performance and nitrogen metabolism in weaned pigs fed diets containing different sources of starch. *Livest Sci* 2007, 109:73-76.

65.   Regmi PR, Metzler-Zebeli BU, Gänzle MG, Van Kempen TA, Zijlstra RT: Starch with high amylose content and low *in vitro* digestibility increases intestinal nutrient flow and microbial fermentation and selectively promotes bifidobacteria in pigs. *J Nutr* 2011, 141:1273-1280.

66.   Drew M, Schafer T, Zijlstra R: Glycemic index of starch affects nitrogen retention in grower pigs. *J Anim Sci* 2012, 90:1233-1241.

67.   Fouhse JM, Gänzle MG, Regmi PR, Van Kempen TA, Zijlstra RT: High amylose starch with low *in vitro* digestibility stimulates hindgut fermentation and has a bifidogenic effect in weaned pigs. *J Nutr* 2015, 145:2464-2470.

68.   Da Silva CS, Bosch G, Bolhuis J, Stappers L, Van Hees H, Gerrits W, Kemp B: Effects of alginate and resistant starch on feeding patterns, behaviour and performance in *ad libitum*-fed growing pigs. *Animal* 2014, 8:1917-1927.

69.   Fouhse JM, Zijlstra RT: Impact of resistant vs. digested starch on starch energy value in the pig gut. *Bioact Carbohydr Dietary Fibre* 2018, 15:12-20.

70.   Vicari T, Van den Borne JJGC, Gerrits WJJ, Zbinden Y, Blum JW: Postprandial blood hormone and metabolite concentrations influenced by feeding frequency and feeding level in veal calves. *Domest Anim Endocrinol* 2008, 34:74-88.

71.   Solomon TPJ, Chambers ES, Jeukendrup AE, Toogood AA, Blannin AK: The effect of feeding frequency on insulin and ghrelin responses in human subjects. *Br J Nutr* 2008, 100:810-819.

72.   Miao M, Jiang B, Cui SW, Zhang T, Jin Z: Slowly digestible starch—A review. *Crit Rev Food Sci Nutr* 2015, 55:1642-1657.

73.   Paredes SP, Van Kempen TATG, Verstegen MWA, Van Hees HMJ, den Hartog LA, Dekker R, Lozinska L, Prykhod'ko O, Pierzynowski S, Gerrits WJJ, et al: Glycaemic response of piglets suffering from growth retardation after birth. In *Unveiling causes for growth retardation*. Edited by Paredes SP. Wageningen 2014: 105-128

74.   Camm E, Martin-Gronert M, Wright N, Hansell J, Ozanne S, Giussani D: Prenatal hypoxia independent of undernutrition promotes molecular markers of insulin resistance in adult offspring. *The FASEB Journal* 2011, 25:420-427.

75.   Herpin P, Le Dividich J, Hulin JC, Fillaut M, De Marco F, Bertin R: Effects of the level of asphyxia during delivery on viability at birth and early postnatal vitality of newborn pigs. *J Anim Sci* 1996, 74:2067-2075.

76.   Brouns F, Kettlitz B, Arrigoni E: Resistant starch and "the butyrate revolution". *Trends Food Sci Technol* 2002, 13:251-261.

77. Mentschel J, Claus R: Increased butyrate formation in the pig colon by feeding raw potato starch leads to a reduction of colonocyte apoptosis and a shift to the stem cell compartment. *Metabolism* 2003, 52:1400-1405.

78. Martin LJ, Dumon HJ, Lecannu G, Champ MM: Potato and high-amylose maize starches are not equivalent producers of butyrate for the colonic mucosa. *Br J Nutr* 2000, 84:689-696.

79. Umu ÖC, Frank JA, Fangel JU, Oostindjer M, Da Silva CS, Bolhuis EJ, Bosch G, Willats WG, Pope PB, Diep DB: Resistant starch diet induces change in the swine microbiome and a predominance of beneficial bacterial populations. *Microbiome* 2015, 3:16.

80. Bird AR, Vuaran M, Brown I, Topping DL: Two high-amylose maize starches with different amounts of resistant starch vary in their effects on fermentation, tissue and digesta mass accretion, and bacterial populations in the large bowel of pigs. *Br J Nutr* 2007, 97:134-144.

81. De Haer L, Merks J: Patterns of daily food intake in growing pigs. *Anim Sci* 1992, 54:95-104.

82. Tolkamp BJ, Kyriazakis I: To split behaviour into bouts, log-transform the intervals. *Anim Behav* 1999, 57:807-817.

83. Powley TL, Phillips RJ: Gastric satiation is volumetric, intestinal satiation is nutritive. *Physiol Behav* 2004, 82:69-74.

84. Schirra J, Göke B: The physiological role of GLP-1 in human: incretin, ileal brake or more? *Regul Pept* 2005, 128:109-115.

85. Black J, Williams B, Gidley M: Metabolic regulation of feed intake in monogastric mammals. In *Voluntary feed intake in pigs*. Wageningen Academic Publishers, Wageningen, Netherlands; 2009: 189-214

86. Benelam B: Satiation, satiety and their effects on eating behaviour. *Nutrition bulletin* 2009, 34:126-173.

87. Maselyne J, Saeys W, Van Nuffel A: Quantifying animal feeding behaviour with a focus on pigs. *Physiol Behav* 2015, 138:37-51.

88. Quiniou N, Dubois S, Le Cozler Y, Bernier J-F, Noblet J: Effect of growth potential (body weight and breed/castration combination) on the feeding behaviour of individually kept growing pigs. *Livest Prod Sci* 1999, 61:13-22.

89. Quiniou N, Dubois S, Noblet J: Voluntary feed intake and feeding behaviour of group-housed growing pigs are affected by ambient temperature and body weight. *Livest Prod Sci* 2000, 63:245-253.

90. De Haer L, De Vries A: Effects of genotype and sex on the feed intake pattern of group housed growing pigs. *Livest Prod Sci* 1993, 36:223-232.

91. Labroue F, Guéblez R, Meunier-Salaün M-C, Sellier P: Feed intake behaviour of group-housed Piétrain and Large White growing pigs. In *Annales de zootechnie*1999: 247-261.

92. Fàbrega E, Tibau J, Soler J, Fernández J, Font J, Carrión D, Diestre A, Manteca X: Feeding patterns, growth performance and carcass traits in group-housed growing-finishing pigs: the effect of terminal sire line, halothane genotype and age. *Anim Sci* 2003, 77:11-21.

93. Nyachoti C, Zijlstra R, De Lange C, Patience J: Voluntary feed intake in growing-finishing pigs: A review of the main determining factors and potential approaches for accurate predictions. *Can J Anim Sci* 2004, 84:549-566.

94. Li Q, Patience JF: Factors involved in the regulation of feed and energy intake of pigs. *Anim Feed Sci Tech* 2017, 233:22-33.

95.   De Haer L, De Vries A: Feed intake patterns of and feed digestibility in growing pigs housed individually or in groups. *Livest Prod Sci* 1993, 33:277-292.

96.   Lopez J, Jesse G, Becker B, Ellersieck M: Effects of temperature on the performance of finishing swine: II. Effects of a cold, diurnal temperature on average daily gain, feed intake, and feed efficiency. *J Anim Sci* 1991, 69:1850-1855.

97.   Becker B, Knight C, Veenhuizen J, Jesse G, Hedrick H, Baile C: Performance, carcass composition, and blood hormones and metabolites of finishing pigs treated with porcine somatotropin in hot and cold environments. *J Anim Sci* 1993, 71:2375-2387.

98.   Lopez J, Jesse G, Becker B, Ellersieck M: Effects of temperature on the performance of finishing swine: I. Effects of a hot, diurnal temperature on average daily gain, feed intake, and feed efficiency. *J Anim Sci* 1991, 69:1843-1849.

99.   Strubbe JH, Van Dijk G: The temporal organization of ingestive behaviour and its interaction with regulation of energy balance. *Neurosci Biobehav Rev* 2002, 26:485-498.

100.   Moore R: Suprachiasmatic Nucleus. *International Encyclopedia of the Social & Behavioral Sciences* 2001:15290 - 15294.

101.   Reppert S, Moore R: *Suprachiasmatic nucleus: the mind's clock.* Oxford University Press, USA; 1991.

102.   Ingram D, Dauncey M: Circadian rhythms in the pig. *Comp Biochem Physiol A Physiol* 1985, 82:1-5.

103.   Bornett H, Morgan C, Lawrence A, Mann J: The effect of group housing on feeding patterns and social behaviour of previously individually housed growing pigs. *Appl Anim Behav Sci* 2000, 70:127-141.

104.   Van Erp RJJ, Van Kempen TATG, de Vries S, Gerrits WJJ: Unpublished data.

105.   Boumans IJ, de Boer IJ, Hofstede GJ, la Fleur SE, Bokkers EA: The importance of hormonal circadian rhythms in daily feeding patterns: An illustration with simulated pigs. *Horm Behav* 2017, 93:82-93.

106.   Tast A, Love RJ, Evans G, Telsfer S, Giles R, Nicholls P, Voultsios A, Kennaway DJ: The pattern of melatonin secretion is rhythmic in the domestic pig and responds rapidly to changes in daylength. *J Pineal Res* 2001, 31:294-300.

107.   Koopmans SJ, Van der Meulen J, Dekker R, Corbijn H, Mroz Z, Group AS: Diurnal rhythms in plasma cortisol, insulin, glucose, lactate and urea in pigs fed identical meals at 12-hourly intervals. *Physiol Behav* 2005, 84:497-503.

108.   Hyun Y, Ellis M, McKeith F, Wilson E: Feed intake pattern of group-housed growing-finishing pigs monitored using a computerized feed intake recording system. *J Anim Sci* 1997, 75:1443-1451.

109.   Hyun Y, Ellis M: Effect of group size and feeder type on growth performance and feeding patterns in finishing pigs. *J Anim Sci* 2002, 80:568-574.

110.   Feddes J, Young B, DeShazer J: Influence of temperature and light on feeding behaviour of pigs. *Appl Anim Behav Sci* 1989, 23:215-222.

111.   Parsons MJ, Moffitt TE, Gregory AM, Goldman-Mellor S, Nolan PM, Poulton R, Caspi A: Social jetlag, obesity and metabolic disorder: investigation in a cohort study. *Int J Obes* 2014, 39:842.

112.   Colles SL, Dixon J, O'brien P: Night eating syndrome and nocturnal snacking: association with obesity, binge eating and psychological distress. *Int J Obes* 2007, 31:1722.

113.   Roenneberg T, Allebrandt KV, Merrow M, Vetter C: Social jetlag and obesity. *Curr Biol* 2012, 22:939-943.

114. Karlsson B, Knutsson A, Lindahl B: Is there an association between shift work and having a metabolic syndrome? Results from a population based study of 27 485 people. *Occup Environ Med* 2001, 58:747-752.

115. Leproult R, Holmbäck U, Van Cauter E: Circadian misalignment augments markers of insulin resistance and inflammation, independently of sleep loss. *Diabetes* 2014:DB_131546.

116. Gan Y, Yang C, Tong X, Sun H, Cong Y, Yin X, Li L, Cao S, Dong X, Gong Y: Shift work and diabetes mellitus: a meta-analysis of observational studies. *Occup Environ Med* 2015, 72:72-78.

117. Morris CJ, Yang JN, Garcia JI, Myers S, Bozzi I, Wang W, Buxton OM, Shea SA, Scheer FA: Endogenous circadian system and circadian misalignment impact glucose tolerance via separate mechanisms in humans. *Proceedings of the National Academy of Sciences* 2015:201418955.

118. Morris CJ, Yang JN, Scheer FA: The impact of the circadian timing system on cardiovascular and metabolic function. In *Prog Brain Res. Volume* 199: Elsevier; 2012: 337-358

119. Ellingsen T, Bener A, Gehani A: Study of shift work and risk of coronary events. *The journal of the Royal Society for the Promotion of Health* 2007, 127:265-267.

120. Cummings DE, Overduin J: Gastrointestinal regulation of food intake. *J Clin Invest* 2007, 117:13-23.

121. Berthoud H-R: The vagus nerve, food intake and obesity. *Regul Pept* 2008, 149:15-25.

122. Verdich C, Flint A, Gutzwiller J-P, Naslund E, Beglinger C, Hellstrom P, Long S, Morgan L, Holst J, Astrup A: A meta-analysis of the effect of glucagon-like peptide-1 (7–36) amide on *ad libitum* energy intake in humans. *J Clin Endocrinol Metab* 2001, 86:4382-4389.

123. Edholm T, Degerblad M, Grybäck P, Hilsted L, Holst JJ, Jacobsson H, Efendic S, Schmidt P, Hellström PM: Differential incretin effects of GIP and GLP-1 on gastric emptying, appetite, and insulin-glucose homeostasis. *Neurogastroenterol Motil* 2010, 22:1191.

124. Jain NK, Boivin M, Zinsmeister AR, Brown ML, Malagelada J-R, DiMagno EP: Effect of ileal perfusion of carbohydrates and amylase inhibitor on gastrointestinal hormones and emptying. *Gastroenterology* 1989, 96:377-387.

125. Ropert A, Cherbut C, Roze C, Le Quellec A, Holst J, Fu-Cheng X, Des Varannes SB, Galmiche J: Colonic fermentation and proximal gastric tone in humans. *Gastroenterology* 1996, 111:289-296.

126. Zhou J, Martin RJ, Tulley RT, Raggio AM, McCutcheon KL, Shen L, Danna SC, Tripathy S, Hegsted M, Keenan MJ: Dietary resistant starch upregulates total GLP-1 and PYY in a sustained day-long manner through fermentation in rodents. *Am J Physiol Endocrinol Metab* 2008, 295:E1160-E1166.

127. Burcelin R: When gut fermentation controls satiety: A PYY story. *Mol Metab* 2017, 6:10-11.

128. Anderson GH, Catherine NL, Woodend DM, Wolever TM: Inverse association between the effect of carbohydrates on blood glucose and subsequent short-term food intake in young men. *Am J Clin Nutr* 2002, 76:1023-1030.

129. Nilsson AC, Östman EM, Holst JJ, Björck IM: Including indigestible carbohydrates in the evening meal of healthy subjects improves glucose tolerance, lowers inflammatory markers, and increases satiety after a subsequent standardized breakfast. *J Nutr* 2008, 138:732-739.

130. Beaulieu A, Williams N, Patience J: Response to dietary digestible energy concentration in growing pigs fed cereal grain-based diets. *J Anim Sci* 2009, 87:965-976.

Chapter I

Chapter 2

*Feed intake patterns nor pig performance
are affected by dietary resistant starch,
despite marked differences in digestion*

Rik J.J. van Erp, Sonja de Vries, Theo A.T.G. van Kempen, Leo A. Den Hartog,
Walter J.J. Gerrits

Abstract

Current feed evaluation systems often assume that fermented starch, i.e., resistant starch (RS), yields less energy than digested starch. However, growth rates of pigs fed low and high RS diets are often the same when feed is available *ad libitum*. This may be explained by its effect on digestive processes changing feeding behavior, and consequently energy utilization. Therefore, this study aims to investigate the effect of RS on nutrient digestion and digesta passage rate in pigs, in combination with its effect on feeding behavior and growth performance under *ad libitum* conditions. In experiment 1, twenty male pigs (34.9±1.36 kg) were fed diets containing either 50% waxy maize starch (low in RS; LRS) or high-amylose maize starch (high in RS; HRS), and soluble and insoluble indigestible markers. After 14 days of adaptation to the diets, pigs were fed hourly to reach steady state (6h), dissected, and digesta were collected from 8 segments. From the collected samples, nutrient digestion and passage rate of the solid and liquid digesta fraction were determined. In experiment 2, 279 pigs (80.6±1.07 kg; sex ratio 1:1) were housed in groups of 6, fed one of both diets, and slaughtered at approximately 115 kg. Feed intake, growth, carcass gain, and slaughter quality parameters were measured. Ileal starch digestibility was greater for LRS-fed than HRS-fed pigs (98.0% vs. 74.0%; P<0.001), the difference being fermented in the large intestine. No difference in passage rate of either the solid or liquid digesta fraction was observed. Growth rate and feed intake were similar for LRS-fed and HRS-fed pigs, whereas feed efficiency of HRS-fed pigs was 1 %-unit greater than that of LRS-fed pigs (P=0.041). The efficiency of feed used for carcass gain, however, was similar for both diets indicating that the difference in feed efficiency was determined by the non-carcass fraction; mainly explained by weight of the gastro-intestinal tract and its contents. Feeding behavior was not affected by starch source. In conclusion, despite a substantial reduction in digestible starch when pigs were fed HRS instead of LRS, carcass gain, slaughter quality parameters, and feed efficiency used for carcass gain were unaffected. Resistant starch did not affect digesta passage rate nor feeding behavior suggesting that the difference in energy intake between fermentable and digestible starch is compensated for post-absorptively. Our results indicate that the net energy value of fermented starch currently used in pig feed evaluation systems is underestimated and should be reconsidered.

2.1 Introduction

The rate and extent of starch digestion in the porcine small intestine varies among botanical sources [2-5]. Regardless its source, however, starch not enzymatically digested, i.e., resistant starch (RS), is typically completely fermented by microbes residing in the gastro-intestinal tract (GIT; [6-8]). In current feed evaluation systems for pigs, the net energy value of fermented RS is often assumed to be 30% lower than that of digested starch; an estimate that is derived by regression of net energy on digestible nutrients [9]. Using indirect calorimetry, however, Gerrits*, et al.* [7] showed the net energy value of fermented, retrograded maize starch to be only 20% lower than that of digestible maize starch in restrictedly fed pigs, while the difference in apparent digestible energy intake was 50%. Under *ad libitum* feeding conditions, growth rates of pigs fed high-RS diets have been reported to be similar to [10, 11], or greater than [12], those of pigs fed low-RS diets. These findings suggest that current feed evaluation systems may underestimate the net energy value of RS.

Replacing digestible starch by RS decreases glucose absorption in the small intestine, while increasing short-chain fatty acids (SCFA) absorption in the large intestine [13]. Furthermore, RS interferes with the digestion of other nutrients [14-17], and changes digesta passage rate [17, 18]. Consequently, RS affects the amount and site of nutrient absorption, and ultimately nutrient utilization. Possibly through these effects, RS impacts feeding motivation [19, 20], which may affect energy metabolism of pigs. Under restricted feeding conditions, RS has been shown to decrease energy losses through reduced physical activity [7, 21, 22], which was suggested to be related to its satiating properties [22]. Under unrestricted feeding conditions, feeding high-RS diets increased meal size of growing pigs, whereas meal frequency was reduced [10]. Furthermore, pigs poorly adapted to high-RS diets decreased their meal size, and consequently feed intake [23]. Alterations in feeding behavior may interact with the decrease in postprandial glucose and insulin responses when RS is replaced for digestible starch [2, 13], as these responses have been shown to be proportional to meal size in humans [24, 25] and calves [26, 27]. Insulin stimulates protein and fat synthesis [28], and plays a major role in the regulation of energy homeostasis [29], which in turn is an important regulator of feed intake [30]. It is, however, unclear if the effect of RS on digestive processes and consequently feeding behavior could explain the similar growth rates of pigs fed RS and digestible starch under *ad libitum* conditions. Therefore, we investigated the effect of exchanging digestible starch for RS on nutrient digestibility and digesta passage rate (experiment 1) in combination with its effect on feeding behavior, growth performance, and feed efficiency under *ad libitum* feeding conditions (experiment 2).

Chapter 2

2.2 Materials & Methods

Table 2.1 Ingredient composition and analyzed chemical composition of experimental diets.

	Diets	
	LRS[3]	HRS[4]
Ingredient (g/kg)		
Waxy maize	500.0	-
High amylose maize	-	497.0
Rapeseed meal	150.0	150.0
Sunflower seed meal	150.0	150.0
Wheat gluten meal	94.5	94.5
Water[1]	25.0	28.0
Molasses, cane	25.0	25.0
Palm oil	21.6	21.6
Premix[2]	12.5	12.5
Calcium Carbonate	8.1	8.1
L-Lysine	5.4	5.4
Sodium Bicarbonate	4.3	4.3
Mono-calcium phosphate	2.7	2.7
Choline Chloride	0.1	0.1
L-Threonine	0.6	0.6
L-Tryptophan	0.1	0.1
Phytase	0.1	0.1
Analyzed chemical composition (g/kg)		
Dry matter	886	873
Crude protein (N × 6.25)	179	181
Starch	420	430
Gross energy, MJ/kg	17.7	17.7
Calculated SID AA[4] (g/kg)		
Isoleucine	5.6	5.6
Leucine	9.9	9.9
Lysine	8.6	8.6
Methionine + Cysteine	5.8	5.8
Threonine	5.2	5.2
Tryptophan	1.6	1.6
Valine	6.4	6.4

[1]Water was included to compensate for a lower dry matter content of high-amylose maize starch. [2]Supplied per kg of feed: Citric acid 111 mg; propyl gallate 69 mg; butyl hydroxy-toluene 151 mg; sepiolite 158 g; vitamin A 8,000 IU; vitamin D3 1600 IU; vitamin E (all-rac-alpha-tocoopheryl acetate) 7.5 IU; vitamin K3 (menadione nicotinamide bisulphate) 160 mg; vitamin B1 (thiamine mononitrate) 80 mg; vitamin B2 (riboflavin) 400 mg; calcium-D-pantothenate 1,3 g; choline chloride 12 g; niacin 1,6 g; vitamin B6 (pyridoxine hydrochloride) 120 mg; folic acid 120 mg; vitamin B12 (cyanocobalamin) 1.6 mg; biotin 12 mg; betaine-hydrochloride 7.9 mg; iron(II) sulphate 8 g; calcium iodate 80 mg; cupper(II) sulphate 12 g; manganese(II) oxide 2.4 g; zinc oxide 8 g; sodium selenite 24 mg. [3]Low resistant starch. [4]High resistant starch. [5]Standardized ileal digestible amino acids.

Experiments were performed at the experimental facilities of Nutreco N.V., Sint Anthonis, the Netherlands.

2.2.1 Experiment 1

Twenty male pigs (34.9±1.36 kg; Hypor Libra x Hypor Maxter, Hendrix Genetics, Boxmeer, the Netherlands) were selected at 11 weeks of age and housed individually in pens of 2.5m x 0.95m with 40% slatted floors, during a period of 16 d. Pigs were exposed to 16 h of light (day 0-14, from 06.00h to 22.00h; day 14-15, from 05.00h to 21.00h) and 8 h of darkness. Temperature was controlled at 23°C. In the first 2 d of the experiment, pigs were gradually switched from a commercial diet (CP 172 g/kg, Net Energy (NE) 9.17 MJ/kg; ABZ, Leusden, Netherlands) to one of two experimental diets; low-RS (LRS), containing 50% of waxy maize starch (Roquette, Lestrem, FR), or high-RS (HRS), containing 50% of high amylose maize starch (Roquette, Lestrem, FR) (Table 2.1). In both diets, titanium dioxide (TiO$_2$; 3 g/kg) and chromium ethylenediaminetetraacetic acid (Cr-EDTA; 1.2 g/kg) were added as indigestible markers to represent the solid (TiO$_2$) and liquid (Cr-EDTA) phases of the digesta. Pigs were fed at 2.3 times maintenance requirements (750 kJ of NE·kg$^{-0.6}$; [31]). Feed allowance was adjusted daily, based on BW and expected daily gain (600 g/d). Water was available *ad libitum* during the entire study period. Pigs were fed twice a day until day 13, after which daily meal frequency was increased to six meals that were equally distributed between 05.30h and 20.30h. At day 16, pigs were hourly fed, with a minimum number of six meals (each meal equaled 1/12 of the daily portion), to approach steady-state marker concentrations in the stomach and small intestine. During this period, lights remained on. Subsequently, pigs were sedated sequentially (one hour after the last meal) by an intramuscular injection of Zoletil® 100 (0.06 ml/kg BW) and an intravenous injection of Euthasol® 20% (24 mg/kg BW) in the ear, after which they were euthanized by exsanguination via the carotid artery. The sequence in which pigs were dissected was blocked to account for the difference in number of meals pigs received before dissection. Each block consisted of two HRS-fed and two LRS-fed pigs.

Dissection

Animals were weighed at the start of the experiment, at the first day of the second week, and before dissection. After euthanasia, the abdominal cavity was opened, and the GIT removed and immediately dissected into eight segments: stomach; small intestine into 3 segments (based on length): first half of the small intestine (proximal SI), second half minus the last 1.5 m of the small intestine (mid SI), last 1.5 m of small intestine (distal SI); caecum; colon into 2 segments (based on length): first half of the colon (proximal colon), second half of the colon (distal colon); and rectum. Before removal of the GIT from the abdominal cavity, surgical clamps were placed at the start and end of each segment. After dissection,

gastro-intestinal (GI) segments, with exception of the rectum, were cleaned from blood using paper towels, and weighed before and after removal of digesta from each segment. The digesta in each segment were removed by gentle squeezing, collected quantitatively, and stored at -20ºC. Before storage, digesta pH was measured by using a Hanna HI99141 pH meter (Hanna, Netherlands) with exception of rectal samples, and homogenous subsamples were taken from digesta collected in the stomach, mid SI, and proximal colon, and stored at 4 ºC, which were subsequently analyzed for viscosity and water-binding capacity (WBC) within 3 days after collection.

Analytical methods and calculations

Water-binding capacity (WBC) of digesta was measured following the procedure described by [32], by centrifuging digesta at 4000 x g for 10 min at 21 °C after which the supernatant was decanted. Water-binding capacity was calculated as gram of water retained per gram dry matter (DM) after decanting. Due to limited sample material for some segments, all WBC analyses were carried out in singlicate. Dynamic viscosity of digesta was measured following a modified procedure described by Shelat*, et al.* [33], using a controlled stress rheometer (MCR502, Anton Paar GmbH, Grass, Austria) with a parallel plate geometry (25 mm; PP25/P2-SN25491, PP25/P2-SN25463; Anton Paar GmbH) by applying a continuous shear rate sweep from 50 to 1 s^{-1} in 25 equally proportioned steps after 30 s pre-shear at 10 s^{-1}. Gap size was set at 1.5 mm for stomach, proximal SI, mid SI, and distal SI, and at 2 mm for proximal colon samples to avoid slip between plate and sample. The amount of sample material used covered the complete surface of the parallel plate; excess material was removed carefully by using a spatula. All viscosity measurements were performed in duplicate at 39 °C. Due to limited sample material one observation (treatment HRS) of the mid SI was missing. The apparent viscosity of digesta samples obtained over the range of shear rates was modelled using a power law model [1].

[1] $\qquad \eta = K \cdot \dot{\gamma}^{n-1}$,

where η = dynamic viscosity, K = flow consistency constant, which equals dynamic viscosity when shear rate is 1, $\dot{\gamma}$ = shear rate in s^{-1}, n = power law or flow behavior index, which determines the shape of the curve.

Prior to chemical analyses, digesta samples from each segment were freeze-dried and ground to pass a 1 mm screen. Digesta samples and diets were analyzed for DM [34], nitrogen according to the Dumas principle (628 Series, Leco Corporation, United States) [35], starch [36], titanium [37], and chromium [38] after sample preparation as described by Williams*, et al.* [39]. All analyses were carried out in duplicate.

Apparent total tract digestibility (ATTD) of nutrients were calculated by using the following equation [40]:

$$[2]\ \text{Nutrient dissappearance (\% of intake)} = \left(1 - \left(\frac{\text{Nutrient}_{\text{digesta}}}{\text{Ti}_{\text{digesta}}} \Big/ \frac{\text{Nutrient}_{\text{feed}}}{\text{Ti}_{\text{feed}}}\right)\right) \times 100$$

where $\text{Nutrient}_{\text{digesta}}$ is the nutrient concentration in the feces (g/kg DM), $\text{Ti}_{\text{digesta}}$ is the titanium concentration in the feces (g/kg DM), $\text{Nutrient}_{\text{feed}}$ is the nutrient concentration in the feed (g/kg DM), and Ti_{feed} is the titanium concentration in the feed (g/kg DM).

Mean retention time (MRT; hour) of solid and liquid phase markers in the stomach, SI1, SI2, and SI3 were calculated using equation 3, assuming that steady-state marker concentrations in the proximal GIT were reached:

$$[3]\ \text{MRT (n)} = \frac{[\text{marker}] \times W}{l}$$

where marker pool sizes in digesta of each segment (n) were calculated from Ti (as TiO_2) or Cr (as Cr-EDTA) concentrations in digesta (g/kg DM) at dissection multiplied by the weight of digesta (W) in the corresponding segment (g DM). Marker intake (l) was calculated by multiplying diet marker concentration (g/kg DM) with hourly feed intake during steady state feeding (g DM).

2.2.2 Experiment 2

In total, 288 pigs (80.6 ±1.07 kg; Hypor Libra x Hypor Maxter, Hendrix Genetics, Boxmeer, the Netherlands) were housed in 48 pens (6 pigs per pen) in one of six departments. Sexes were equally divided over pens (1:1, male:female). Pens (4.70 x 2.40 m) had 60% slatted floors and were equipped with an electronic single space feeding station (EFS) for fattening pigs (Schauer, Prambachkirchen, Austria). At the start of the experimental period, pens were allocated to one of two diets (HRS or LRS), which had the same dietary composition as the diets used in experiment 1 (Table 2.1). Each pig received an electronic ear transponder corresponding to a unique identification number that was read by the electronic feeding station. Data generated by the feeding stations were continuously stored: the pig's identification number, the date, the time of entry and exit per visit, and amount of feed consumed per visit. Meal size, duration, frequency, and intermeal interval were calculated using an average meal criterion of 330 seconds that was estimated via the method of Tolkamp and Kyriazakis [41]. If the interval between two successive visits exceeded the meal criterion, the second visit was considered the start of a new meal. Meal size and duration were calculated as the respective sum of feed intake and visit duration within one meal. Intermeal interval was calculated as the average time in between meals. Rate of feed

intake (RFI) was calculated as feed intake divided by visit duration. Exclusion criteria for cleaning the raw EFS data were based on visual plots of visit time × feed intake, and visit time × rate of feed intake (RFI) and were set to: visit time < 100 s and feed intake > 200 g; visit time > 100 s and rate of feed intake (RFI) > 400 g/min; visit time > 360 s and feed intake < 40 g. Applying these criteria, 976 out of 682638 observations were discarded. Animals were exposed to 16 h of light (from 06.00h to 22.00h) and 8 h of darkness. Temperature was maintained at 21°C. Pigs were weighed at the start and at the end of the experiment, and were slaughtered at 115 kg BW, after which back-fat thickness, meat percentage, loin muscle depth, and carcass weight were determined in the slaughter house (Vion Food Group, Netherlands). Back-fat thickness, meat percentage, and loin muscle depth were measured between the third and fourth rib (from last rib position), six cm from the dorsal mid line using the Capteur Gras/Maigre instrument (GCM; Sydel, France; [42]). Individual daily carcass gain was calculated as the difference between the final carcass weight and estimated initial carcass weight divided by the number of days in experiment. Initial carcass weight was calculated as start weight × the average ratio carcass and body weight of LRS-fed pigs in experiment 1 (0.84). Observations of nine pigs were discarded: five suffered from lameness (1 LRS; 4 HRS), five pigs died (1 LRS; 1 HRS) or lost weight (3 HRS) for unknown reasons.

2.2.3 Data analysis

For all statistical analyses, SAS 9.4 for Windows (SAS Institute, Cary, NC) was used. For experiment 1, the pig was considered as experimental unit. All data of experiment 1 were analyzed using a general linear mixed model (PROC MIXED),

[4] $Y_{ijk} = \mu + D_i + B_j + e_{ijk}$,

where Y is the dependent variable, μ the overall mean, D_i the fixed effect of diet (LRS or HRS), B_j the random effect of block effect dissection order (1-5), and e_{ijk} the residual error. Full and empty intestinal weight, and digesta weight were expressed as g/kg BW.
For experiment 2, the pen (i.e., a group of 6 pigs) was considered as experimental unit. All data of experiment 2 were analyzed using a general linear mixed model (PROC MIXED),

[5] $Y_{ijklm} = \mu + D_i + S_j + P_k + B_l + e_{ijklm}$,

where Y is the dependent variable, μ the overall mean; D_i the fixed effect of diet (LRS or HRS), S_j the fixed effect of sex; P_k the random effect of pen; B_l the random effect of department; and e_{ijklm} the residual error. Average daily feed intake, average daily gain, carcass

gain, were corrected for expected differences related to variation in body weight by adding initial body weight as covariate to the model. Meat percentage, loin muscle depth, and back-fat thickness were corrected for expected differences related to variation in body weight by adding carcass weight as covariate to the model. Interaction between fixed effects diet and sex was checked, and deleted from the model when not significant (P<0.05).

Homogeneity and normality of model residuals of both models were checked visually using UNIVARIATE procedure. Data are presented as LSmeans ± pooled SEM. Differences were considered significant if P < 0.05.

2.3 Results

2.3.1 Experiment 1

All pigs remained healthy during the entire experiment. Body weight (BW ± SD) at the start (34.9 ± 1.36 kg) and end (47.5 ± 2.83 kg) of the experiment, and average daily gain (847 ± 196 g/d) did not differ between diets. After removal of digesta, the caecum, proximal colon, and distal colon of HRS-fed pigs were heavier than those of LRS-fed pigs, 50% (P=0.01), 35% (P<0.01), and 14% (P=0.05), respectively; resulting in a 6% greater gastro-intestinal weight for HRS-fed pigs compared with LRS-fed pigs (P=0.05; Table 2.2). The amount of digesta collected from the caecum and proximal colon was greater for HRS-fed pigs than LRS-fed pigs, 68% (P<0.01) and 78% (P<0.01), respectively; resulting in 25% more digesta collected from the GIT of HRS-fed pigs (P<0.01).

Apparent nutrient digestibility and mean retention time of digesta

Apparent DM digestibility measured at the distal SI was 14.6 %-units smaller for HRS-fed pigs than LRS-fed pigs (P<0.01), whereas apparent DM digestibility measured at the stomach, proximal SI, and mid SI did not differ between diets in (Table 2.3). Apparent DM digestibility measured at the caecum, proximal colon, and distal colon was lower for HRS-fed pigs than LRS-fed pigs (P<0.01). Consequently, apparent total tract digestibility (ATTD) of DM was 3.1 %-units lower for HRS-fed pigs than LRS-fed pigs (P<0.01). Starch digestibility was lower for HRS-fed pigs than LRS-fed pigs measured at the mid SI (by 16 %-units; P<0.01), at the distal SI (by 24 %-units; P<0.01), and total tract (by 0.1 %-unit; P=0.02). Apparent total tract nitrogen (N) digestibility was 7.8 %-units lower for HRS-fed pigs than LRS-fed pigs (P<0.01). Apparent DM digestibility measured at the distal SI corrected for starch and protein losses (N × 6.25) did not differ between diets (LRS: 79.5% vs. HRS: 77.6 %; P=0.16). Mean retention times of solid and liquid phases of digesta in the stomach and small intestine were not affected by starch source (Table 2.3). Regardless of dietary treatment, MRT of the solid phase in the stomach was 30 min longer than that of

the liquid phase (P=0.03), whereas MRT of the solid (2.03 h) and liquid phase (1.96 h) in the small intestine did not differ.

Table 2.2 Empty, full, and digesta weight of different segments of the gastro-intestinal tract of growing male pigs (48±2.8 kg) fed either a diet containing 50% waxy maize starch (low resistant starch; LRS) or 50% high amylose maize starch (high resistant starch; HRS) during experiment 1.[1]

Weight (g/kg BW)	Full				Empty				Digesta			
	LRS	HRS	SEM	P^5	LRS	HRS	SEM	P^5	LRS	HRS	SEM	P^5
Stomach	34.1	37.9	2.3	0.26	6.6	6.5	0.2	0.64	27.5	31.4	2.3	0.25
Proximal SI[2]	21.6	20.9	1.0	0.63	16.3	15.7	0.6	0.55	5.3	5.2	0.7	0.88
Mid SI[3]	26.5	24.4	1.6	0.38	13.3	12.9	0.5	0.53	13.2	11.5	1.5	0.44
Distal SI[4]	8.4	8.4	0.6	0.98	4.2	4.1	0.2	0.79	4.2	4.3	0.6	0.94
Caecum	7.0	11.4	0.7	<0.01	2.0	3.0	0.2	0.01	5.0	8.4	0.5	<0.01
Proximal colon	23.5	38.3	2.0	<0.01	8.2	11.1	0.4	<0.01	15.3	27.2	1.8	<0.01
Distal colon	14.2	17.5	1.4	0.10	4.9	5.6	0.2	0.05	9.3	11.9	1.3	0.16
Total	135	159	4.6	<0.01	55	58	1.1	0.03	80	100	4.7	<0.01
Total (% of BW)	13.7	15.7	0.3	<0.01	5.6	5.9	0.1	0.05	8.1	9.9	0.3	<0.01

[1]Data are presented as LS means ± pooled SEM; n=10 per treatment, with individual pig as the experimental unit. [2]First half of small intestine. [3]Second half of small intestine minus final 1.5m. [4]Final 1.5m of small intestine. [5]Model established p-values for fixed effect of diet.

Physicochemical properties of digesta

Apparent digesta viscosity measured at a shear rate of 45·s tended to be greater for HRS-fed pigs in the stomach (P=0.06), and was greater for HRS-fed pigs than LRS-fed pigs in the proximal colon (5.48 vs. 2.43 mPA·s; P=0.01; Table 2.4). The flow consistency constant of the digesta (K) was greater for HRS-fed pigs than LRS-fed pigs in the stomach (47.5 vs. 35.3; P=0.035), and proximal colon (148 vs. 46.3; P<0.01). The power law index n of the digesta in the proximal colon was lower for HRS-fed pigs than LRS-fed pigs (0.14 vs. 0.22; P<0.01). Dry matter content of the digesta was greater in the distal SI (by 33 g/kg; P=0.03) and caecum (by 31 g/kg; P=0.01) for HRS-fed pigs than LRS-fed pigs, and lower in the distal colon (by 41 g/kg; P=0.05) and rectum (by 62 g/kg; P=0.02; Table 2.5). Water-binding capacity of the digesta was greater for HRS-fed pigs than LRS-fed pigs in all GI segments measured (P<0.05). Digesta pH was not affected by starch source in any of the GI segments measured.

Table 2.3 Apparent digestibility of dry matter, nitrogen, and starch, and mean retention time of solid (TiO$_2$) and liquid phase (Cr-EDTA) markers in different segments of the gastro-intestinal tract of growing male pigs (48±2.8 kg) fed a diet either containing 50% waxy maize starch (low resistant starch; LRS) or 50% of high amylose maize starch (high resistant starch; HRS) during experiment 1.[1]

| | Apparent digestibility (%) | | | | | | | | | | | | Mean retention time (h)[5] | | | | | | | |
| | Dry matter | | | | Nitrogen | | | | Starch | | | | Solid phase | | | | Liquid phase | | | |
	LRS	HRS	SEM	p	LRS	HRS	SEM	p	LRS	HRS	SEM	p	LRS	HRS	SEM	p	LRS	HRS	SEM	p
Stomach	-0.7	5.2	2.5	0.10	4.8	9.1	3.1	0.32	-	-	-	-	2.1	2.4	0.2	0.32	1.6	1.9	0.2	0.39
Proximal SI[2]	18	22	4.5	0.47	14	20	5.0	0.40	41	36	4.6	0.55	0.3	0.4	0.1	0.60	0.3	0.3	0.1	0.83
Mid SI[3]	57	51	2.7	0.19	53	54	4.4	0.81	85	69	2.6	<0.01	1.2	1.1	0.2	0.71	1.1	1.1	0.1	0.94
Distal SI[4]	73	59	2.0	<0.01	75	69	2.6	0.13	98	74	1.1	<0.01	0.6	0.5	0.1	0.35	0.5	0.5	0.1	0.56
Caecum	78	71	0.9	<0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Proximal colon	83	76	0.5	<0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Distal colon	85	81	0.4	<0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rectum	84	81	0.6	<0.01	84	76	1.2	<0.01	100	100	0.5	0.02	-	-	-	-	-	-	-	-

[1]Data are presented as LS means ± SEM, n=10 for both treatments, with each experimental unit being one pig. [2]First half of small intestine. [3]Second half of small intestine minus last 1.5 m. [4]Last 1.5m of small intestine. [5]Mean retention time for both solid and liquid phase markers were calculated by assuming that steady state marker concentrations in the proximal gastro-intestinal tract were reached.

Chapter 2

Table 2.4 Flow consistency constant K, flow behavior index n, and apparent viscosity of digesta collected from different segments of the gastro-intestinal tract of growing male pigs (48±2.8 kg) fed a diet containing 50% waxy maize starch (low resistant starch; LRS) or 50% high amylose maize starch (high resistant starch; HRS) during experiment 1.[1]

	$K^{4,5,6}$				$n^{4,5,6}$				Apparent viscosity (mPA·s)^{4,5,6}			
	LRS	HRS	SEM	P	LRS	HRS	SEM	P	LRS	HRS	SEM	P
Stomach	35.3	47.5	4.69	0.04	0.03	0.03	0.02	0.84	0.89	1.20	0.12	0.06
Mid SI^{2,3}	44.6	52.9	10.5	0.56	0.07	0.04	0.03	0.34	1.24	1.23	0.22	0.97
Proximal colon	46.3	148	18.7	<0.01	0.22	0.14	0.01	<0.01	2.43	5.48	0.76	0.01

[1]Data are presented as LS means ± SEM, n=10 per treatment, unless indicated otherwise, with each experimental unit being one pig. [2]Second half minus final 1.5m of small intestine. [3]n = 9 for mid SI due to limited sample material from one pig. [4]Measured using a controlled stress rheometer at 39 °C with a parallel plate geometry (25 mm) by applying a continuous shear rate sweep from 50 to 1 s^{-1} in 25 equally proportioned steps after 30 s pre-shear at 10 s^{-1}. Gap size was set at 1.5 mm for stomach, proximal SI, mid SI, and distal SI, and at 2 mm for proximal colon samples. [5]Determined using the power law index $\eta = K \cdot \dot{\gamma}^{n-1}$ [1]. [6]Measured at a shear rate of 45·s.

Table 2.5 Dry matter, pH, and water-binding capacity of digesta collected from different segments of the gastro-intestinal tract of growing male pigs (48±2.8 kg) fed a diet containing 50% waxy maize starch (low resistant starch; LRS) or 50% high amylose maize starch (high resistant starch; HRS) during experiment 1.[1]

	Dry matter (g/kg)				pH				Water-binding capacity (g/g)			
	LRS	HRS	SEM	P	LRS	HRS	SEM	P	LRS	HRS	SEM	P
Stomach	211	198	9.5	0.37	4.15	4.27	0.17	0.59	0.96	1.09	0.03	<0.01
Proximal SI[2]	162	163	11.7	0.96	5.51	5.63	0.13	0.80	-	-	-	-
Mid SI[3]	115	134	10.1	0.20	6.38	6.31	0.14	0.74	1.49	1.88	0.13	0.05
Distal SI[4]	109	142	10.2	0.03	6.67	6.84	0.12	0.32	-	-	-	-
Caecum	120	151	7.8	0.01	5.69	5.52	0.09	0.18	3.25	3.96	0.16	<0.01
Proximal colon	203	187	11.6	0.29	6.09	5.91	0.15	0.13	3.25	4.24	0.17	<0.01
Distal colon	303	262	13.9	0.05	6.55	6.48	0.13	0.71	2.31	2.83	0.17	0.04
Rectum	352	290	17.4	0.02	-	-	-	-	-	-	-	-

[1]Data are presented as LS means ± SEM, n=10 per treatment, unless indicated otherwise, with each experimental unit being one pig. [2]First half of small intestine. [3]Second half minus final 1.5m of small intestine. [4]Final 1.5m of small intestine.

Table 2.6 Daily feed intake, daily gain, feed efficiency, slaughter quality parameters, and feed intake behavior of finishing pigs fed a diet containing either 50% waxy maize starch (low resistant starch; LRS) or 50% high amylose maize starch (high resistant starch; HRS) for 35 days during experiment 2.[1]

	LRS	HRS	SEM	P-value	
				Diet	Sex[6]
Start weight	80.7	80.4	1.07	0.85	<0.01
End weight	116	116	1.10	0.77	<0.01
Daily gain, g/d[2]	1006	1025	26.2	0.39	<0.01
Feed intake, g/d[2]	2812	2791	63.1	0.70	0.92
Feed efficiency, %[3]	35.8	36.8	0.35	0.04	<0.01
Slaughter quality parameters					
Carcass weight, kg	91.2	90.2	0.85	0.45	<0.01
Non-carcass weight, kg	24.7	26.1	0.36	0.01	<0.01
Carcass gain, g/d[2,4]	669	647	24.4	0.24	<0.01
Non-carcass gain, g/day[2,4]	337	378	8.63	<0.01	<0.01
Feed efficiency based on carcass weight, %	23.8	23.3	0.60	0.34	<0.01
Dressing, % of live weight	78.8	77.6	0.24	<0.01	<0.01
Meat, %[5]	58.5	58.4	0.25	0.43	<0.01
Back-fat thickness, mm[5]	14.9	15.1	0.25	0.43	<0.01
Loin muscle depth, mm[5]	66.0	65.7	0.44	0.63	<0.01
Feeding behavior					
Meal frequency, n/d	12.1	11.9	0.30	0.72	0.02*
Meal size, g	274	262	7.97	0.30	<0.01*
Meal duration, min	8.08	7.78	0.19	0.25	<0.01
Intermeal interval, min	117	114	2.87	0.44	0.03
Time spent eating, min	70.4	69.5	1.39	0.58	0.03
Visit frequency, n/d	42.1	42.1	1.80	0.99	<0.01
Rate of feed intake, g/min	41.3	41.4	1.12	0.93	<0.01

[1]Data are presented as LS means ± SEM, n=24 for both treatments (6 pigs/replicate; sex ratio 1:1), except for seven pens containing five pigs (2 LRS; 5 HRS) and one pen (HRS) containing four pigs. [2]Values are adjusted for expected differences in body weight by including initial body weight as covariate in the statistical model. [3]Calculated as feed to gain ratio in percentage. [4]Calculated using an estimated initial carcass ratio; start weight × the average ratio between carcass and body weight of LRS-fed pigs in experiment 1 (0.84). [5]Values are adjusted for expected differences in body weight by including carcass weight as covariate in the statistical model. [6]Asterisk represents interaction diet × sex (*P*<0.05).

2.3.2 Experiment 2

Body weight did not differ between diets, and averaged 80.6 kg ± 1.07 kg at the start and 116 kg ± 1.10 kg at the end of the experiment. Average daily gain and feed intake were similar for both diets (Table 2.6). Feed efficiency was 1 %-unit greater for HRS-fed pigs than LRS-fed pigs (*P*=0.04). Carcass weight, -gain, and -efficiency, back-fat thickness, meat percentage, and loin muscle depth were similar for both diets (Table 2.6). Dressing percentage was 1.2 %-units smaller for HRS-fed pigs than LRS-fed pigs (*P*<0.01). No

differences in feeding behavior between diets were observed (Table 2.6). The difference in meal size and frequency between male and female pigs (males had less but larger meals), however, was greater in LRS-fed pigs than HRS-fed pigs (diet×sex; $P<0.05$).

2.4 Discussion

It is commonly assumed that starch fermentation yields less net energy than starch digestion [43]. It follows that, exchanging digestible with RS is expected to compromise pig performance. In the current study, however, a substantial increase in RS intake, while reducing apparent total tract DM digestibility by 3 %, did not affect growth performance of finishing pigs. These results are in line with earlier findings in pigs fed high- and low-RS diets [10, 11]. We hypothesized that RS affects digestive processes and consequently feeding behavior of pigs, which may affect pig performance under *ad libitum* conditions.

2.4.1 Nutrient disappearance and mean retention time

Ileal starch disappearance was 24 %-units greater in LRS-fed pigs than in HRS-fed pigs. A minor fraction of the starch escaping small intestinal digestion was excreted with feces, resulting in more starch fermented in the large intestine of HRS-fed pigs (26%) than LRS-fed pigs (2%). These findings are in line with earlier studies, where 12 to 44 % of total starch intake was fermented when feeding high amylose maize starch to ileal cannulated pigs [44-46]. Fecal protein losses (N × 6.25) were 10 %-units greater for HRS-fed pigs than LRS-fed pigs, and accounted for ~50% of the difference in ATTD of DM between diets. Most likely, increased microbial starch fermentation in HRS-fed pigs stimulated formation of microbial biomass. Consequently, ammonia absorption from the large intestine decreases, and urea excretion from blood into the large intestine increases [47, 48], ultimately increasing fecal N excretion. When correcting ATTD of DM for protein (N × 6.25) and starch disappearance, ATTD of DM remained reduced in HRS-fed pigs compared with LRS-fed pigs (85.5 vs. 87.0%; $P=0.010$) possibly indicating that more microbial biomass was excreted by HRS-fed pigs. These results, however, may as well be explained by preferential fermentation of RS over other fermentable dietary fibers thereby increasing fecal excretion of unfermented fibers in HRS-fed pigs [6, 16, 17, 49]. Mean retention time of solid and liquid digesta in the stomach and small intestine were not affected by RS. These results are in contrast with findings of de Vries, *et al.* [17] who observed a 40 min shorter MRT of solid digesta in the upper GIT (time to ileum) of pigs fed retrograded tapioca (high-RS) than of pigs fed native maize starch (low-RS). Ileal DM digestibility, however, was 15 %-units lower in pigs fed retrograded tapioca [17] than pigs fed high-amylose maize starch (this study) increasing ileal DM flow, which is suggested to decrease MRT in the small intestine through its volume effect [50, 51]. Furthermore, physical properties [52] and digestion [17, 46] vary among RS

sources thereby affecting digesta composition and properties along the GIT, and consequently its effect on digesta passage rate. In our study, however, differences between water binding capacity and viscosity of the digesta in the stomach and small intestine of LRS-fed and HRS-fed pigs were minor, and possibly too small to affect MRT of solid and liquid digesta.

2.4.2 Feeding behavior

Replacing digestible starch with RS in the diet did not affect feeding behavior, which is in contrast with Da Silva, *et al.* [10] who reported a greater meal size and lower meal frequency in pigs fed retrograded tapioca (high-RS) than pigs fed pre-gelatinized potato starch (low-RS). Da Silva, *et al.* [10] suggested that a greater glucose appearance and insulin response stimulated short-term satiety reducing meal size in low RS fed pigs, whereas a greater absorption of SCFA prolonged the energy supply to the body delaying satiety signals, consequently decreasing meal frequency in high RS fed pigs. Da Silva, *et al.* [53] showed that glucose and insulin plasma responses were reduced but not delayed in pigs fed retrograded tapioca, indicating that the difference in glucose appearance between these starches is explained by its extent of digestion rather than by its rate. The extent of high amylose maize starch digestion in vivo (73%), however, is greater compared to retrograded tapioca (54%; [17]). Consequently, the short-term effect of glucose on satiety may have been greater in our study compared to Da Silva, *et al.* [10], whereas the long-term effect of microbial short-chain fatty acid production on satiety may have been reduced. In addition, the capacity to digest starch increases with age [54], which may have further reduced the contrast between diets in the second experiment of our study (80-115 kg BW) compared to Da Silva, *et al.* [10] (35 – 115 kg BW). However, the difference in ileal starch digestion between diets observed in experiment 1 was substantial (24%), and changes in starch digestibility with age have been shown to be minor (0.01 %/d; [23]). Therefore, our results may as well suggest that the satiety-regulating role of glucose and insulin, which respective appearance and release are affected by the extent of starch digestion [2, 13], may be less important than previously assumed.

2.4.3 Energy efficiency

Despite a lower enzymatic starch digestibility in HRS-fed pigs, average daily gain, based on live weight and carcass weight, did not differ between diets. On the contrary, feed efficiency was improved. The absence of an effect of starch source on the efficiency of feed used for carcass gain indicates that the effect on feed efficiency was explained by the non-carcass fraction, which was 1.4 kg greater for HRS-fed pigs than for LRS-fed pigs. The latter is most likely explained by a heavier GIT, as full GIT weight was 1 kg greater for HRS-fed than LRS-fed pigs in experiment 1. These results are in line with Bolhuis, *et al.* [55] who observed

a 1.3 kg greater full GIT weight in pigs fed native pea starch than in pigs fed pregelatinized pea starch. Of the total GIT, only the large intestine of HRS-fed pigs was heavier than those of LRS-pigs, of which both organ and digesta weight were increased. The similar efficiency of feed used for carcass gain suggests that net energy efficiency for carcass gain was similar for LRS-fed and HRS-fed pigs, which is in line with Da Silva, *et al.* [10] who observed identical carcass gain : digestible energy intake ratios in pigs fed high and low RS-diets. Visceral fat, however, may have been greater in LRS-fed pigs than HRS-fed pigs, as was shown in mice fed resistant starch diets [56], resulting in an underestimation of feed efficiency for carcass gain in LRS-fed pigs; maximum 0.5 %-unit, based on 0.5 kg visceral fat that can be calculated from the difference in non-carcass weight (1.5 kg; experiment 2) and the difference in GIT weight (1 kg; experiment 1) between treatments. Nevertheless, our results suggest that the net energy of fermented and digested starch are more similar than currently assumed; fermented starch yields 30% less net energy than digested starch [9]. In contrast to our calculations, however, this assumption is based on results obtained in restrictedly-fed pigs. Under restricted conditions, digestible starch stimulates postprandial *de novo* fatty acid synthesis resulting in a greater fat deposition compared to RS, whereas protein deposition is unaffected [7]. In contrast, under unrestricted conditions, fat deposition is continuously stimulated due to a greater and more gradual uptake of dietary glucose [6]. Resistant starch intake increases microbial production of short-chain fatty acids, which are gradually absorbed and available during the day. Consequently, dietary glucose or short-chain fatty acids may be saved from oxidation increasing postprandial fat deposition in RS-fed pigs when feed is available *ad libitum*.

2.5 Conclusions

High RS intake reduced starch digestibility in the small intestine and increased starch fermentation, but did not affect the efficiency of feed used for carcass gain in *ad libitum*-fed pigs. Because RS intake did not affect digesta passage rate nor feeding behavior, the difference in digestible energy intake between digested and fermented starch, must be compensated for post-absorptively. In conclusion, pigs are able to compensate for the lower digestible energy intake with high-RS diets, which suggests that the net energy value of RS currently used in pig feed formulation is underestimated and should be reconsidered.

References

1. Holdsworth S: Applicability of rheological models to the interpretation of flow and processing behaviour of fluid food products. *J Texture Stud* 1971, 2:393-418.

2. Van Kempen TA, Regmi PR, Matte JJ, Zijlstra RT: *In vitro* starch digestion kinetics, corrected for estimated gastric emptying, predict portal glucose appearance in pigs. *J Nutr* 2010, 140:1227-1233.

3. Champ MM-J: Physiological aspects of resistant starch and *in vivo* measurements. *J AOAC Int* 2004, 87:749-755.

4. Solà-Oriol D, Van Kempen T, Torrallardona D: Relationships between glycaemic index and digesta passage of cereal-based diets in pigs. *Livest Sci* 2010, 134:41-43.

5. Li T, Dai, QZ, Yin, YL, Zhang, J, Huang, R, Ruan, Z, Deng, Z, Xie, M: Dietary starch sources affect net portal appearance of amino acids and glucose in growing pigs. *Animal* 2008, 2:723-729.

6. Van Erp RJJ, Van Hees HMJ, Zijlstra RT, Van Kempen TATG, Van Klinken JB, Gerrits WJJ: Reduced feed intake, rather than increased energy losses, explains variation in growth rates of normal-birth-weight piglets. *J Nutr* 2018, 148:1794-1803.

7. Gerrits WJ, Bosch MW, Van den Borne JJ: Quantifying resistant starch using novel, *in vivo* methodology and the energetic utilization of fermented starch in pigs. *J Nutr* 2012, 142:238-244.

8. Fouhse JM, Gänzle MG, Regmi PR, Van Kempen TA, Zijlstra RT: High amylose starch with low *in vitro* digestibility stimulates hindgut fermentation and has a bifidogenic effect in weaned pigs. *J Nutr* 2015, 145:2464-2470.

9. Blok MC, Brandsma G, Bosch G, Gerrits WJJ, Jansman AJM, Fledderus J, Everts H: A new dutch net energy formula for feed and feedstuffs for growing and fattening pigs. In *CVB-Documentation Report*. Wageningen, the Netherlands: Centraal Veevoeder Bureau; 2015.

10. Da Silva CS, Bosch G, Bolhuis J, Stappers L, Van Hees H, Gerrits W, Kemp B: Effects of alginate and resistant starch on feeding patterns, behaviour and performance in *ad libitum*-fed growing pigs. *Animal* 2014, 8:1917-1927.

11. Doti S, Suárez-Belloch J, Latorre M, Guada J, Fondevila M: Effect of dietary starch source on growth performances, digestibility and quality traits of growing pigs. *Livest Sci* 2014, 164:119-127.

12. Van Kempen T, Pujol S, Tibble S, Balfagon A: *In vitro* characterization of starch digestion and its implications for pigs. In *Paradigms in Pig Science*. Edited by Wiseman J, Varley M, Mcorist S, Kemp B. Nottingham: Nottingham University Press; 2007: 515-525

13. Regmi PR, Metzler-Zebeli BU, Gänzle MG, Van Kempen TA, Zijlstra RT: Starch with high amylose content and low *in vitro* digestibility increases intestinal nutrient flow and microbial fermentation and selectively promotes bifidobacteria in pigs. *J Nutr* 2011, 141:1273-1280.

14. Yin F, Zhang Z, Huang J, Yin Y: Digestion rate of dietary starch affects systemic circulation of amino acids in weaned pigs. *Br J Nutr* 2010, 103:1404-1412.

15. Sun T, Lærke HN, Jørgensen H, Knudsen KEB: The effect of extrusion cooking of different starch sources on the *in vitro* and *in vivo* digestibility in growing pigs. *Anim Feed Sci Tech* 2006, 131:67-86.

16. Martínez-Puig D, Castillo M, Nofrarias M, Creus E, Pérez JF: Long-term effects on the digestive tract of feeding large amounts of resistant starch: A study in pigs. *J Sci Food Agric* 2007, 87:1991-1999.

17. de Vries S, Gerrits WJJ, Kabel MA, Vasanthan T, Zijlstra RT: β-Glucans and resistant starch alter the fermentation of recalcitrant fibers in growing pigs. *PLoS One* 2016, 11:e0167624.

18.   Mathers J, Smith H, Carter S: Dose–response effects of raw potato starch on small-intestinal escape, large-bowel fermentation and gut transit time in the rat. *Br J Nutr* 1997, 78:1015-1029.

19.   da Silva CS, Van den Borne JJ, Gerrits WJ, Kemp B, Bolhuis JE: Effects of dietary fibers with different physicochemical properties on feeding motivation in adult female pigs. *Physiol Behav* 2012, 107:218-230.

20.   Bolhuis JE, Van den Brand H, Bartels AC, Oostindjer M, Van den Borne JJ, Kemp B, Gerrits WJ: Effects of fermentable starch on behaviour of growing pigs in barren or enriched housing. *Appl Anim Behav Sci* 2010, 123:77-86.

21.   Schrama JW, Bakker GC: Changes in energy metabolism in relation to physical activity due to fermentable carbohydrates in group-housed growing pigs. *J Anim Sci* 1999, 77:3274-3280.

22.   Bolhuis JE, Van den Brand H, Staals STM, Zandstra T, Alferink SJJ, Heetkamp MJW, Gerrits WJJ: Effects of fermentable starch and straw-enriched housing on energy partitioning of growing pigs. *Animal* 2008, 2:1028-1036.

23.   Van Erp RJJ, Kempen Tv, de Vries S, Gerrits WJJ: Pigs ferment enzymatically digestible starch when it is substituted for resistant starch *J Nutr* 2019, Accepted for publication.

24.   Galgani J, Aguirre C, Díaz E: Acute effect of meal glycemic index and glycemic load on blood glucose and insulin responses in humans. *Nutrition journal* 2006, 5:22.

25.   Brand-Miller J, Thomas M, Swan V, Ahmad Z, Petocz P, Colagiuri S: Physiological validation of the concept of glycemic load in lean young adults. *J Nutr* 2003, 133:2728-2732.

26.   Vicari T, Van den Borne JJGC, Gerrits WJJ, Zbinden Y, Blum JW: Postprandial blood hormone and metabolite concentrations influenced by feeding frequency and feeding level in veal calves. *Domest Anim Endocrinol* 2008, 34:74-88.

27.   Solomon TPJ, Chambers ES, Jeukendrup AE, Toogood AA, Blannin AK: The effect of feeding frequency on insulin and ghrelin responses in human subjects. *Br J Nutr* 2008, 100:810-819.

28.   Saltiel AR, Kahn CR: Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 2001, 414:799.

29.   Brüning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, Klein R, Krone W, Müller-Wieland D, Kahn CR: Role of brain insulin receptor in control of body weight and reproduction. *Science* 2000, 289:2122-2125.

30.   Strubbe JH, Van Dijk G: The temporal organization of ingestive behaviour and its interaction with regulation of energy balance. *Neurosci Biobehav Rev* 2002, 26:485-498.

31.   Everts H: Energy requirement for maintenance in growing pigs. In *CVB-Documentation Report*. Wageningen, The Netherlands: Centraal Veevoeder Bureau; 2015.

32.   Schop M, Jansman AJ, de Vries S, Gerrits WJ: Increasing intake of dietary soluble nutrients affects digesta passage rate in the stomach of growing pigs. *Br J Nutr* 2018:1-9.

33.   Shelat KJ, Nicholson T, Flanagan BM, Zhang D, Williams BA, Gidley MJ: Rheology and microstructure characterisation of small intestinal digesta from pigs fed a red meat-containing Western-style diet. *Food Hydrocolloids* 2015, 44:300-308.

34.   ISO 6496: *ISO 6496: Animal feeding stuffs - Determination of moisture and other volatile matter content.* Geneva, Switzerland: International Organization for Standardization; 1999.

35.   ISO 16634-1:2008: *ISO 16634-1:2008: Food products - Determination of the total nitrogen content by combustion according to the Dumas principle and calculation of the crude protein content -- Part 1: Oilseeds and animal feeding stuffs.* Geneva, Switzerland: International Organization for Standardization; 2008.

Chapter 2

36.   ISO 15914: *ISO 15914: Animal feeding stuffs - Determination of total starch content*. Geneva, Switzerland: International Organization for Standardization 2004.

37.   Myers W, Ludden P, Nayigihugu V, Hess B: A procedure for the preparation and quantitative analysis of samples for titanium dioxide. *J Anim Sci* 2004, 82:179-183.

38.   Van Bussel W, Kerkhof F, Van Kessel T, Lamers H, Nous D, Verdonk H, Verhoeven B, Boer N, Toonen H: Accurate determination of titanium as titanium dioxide for limited sample size digestibility studies of feed and food matrices by inductively coupled plasma optical emission spectrometry with real-time simultaneous internal standardization. *Atom Spectrosc* 2010, 31:81-88.

39.   Williams C, David DJ, Iismaa O: The determination of chromic oxide in faeces samples by atomic absorption spectrophotometry. *J Agric Sci* 1962, 59:381-385.

40.   Kotb A, Luckey T: Markers in nutrition. In *Nutr Abstr Rev*1972: 813-845.

41.   Tolkamp BJ, Kyriazakis I: To split behaviour into bouts, log-transform the intervals. *Anim Behav* 1999, 57:807-817.

42.   Lambooij E, Engel B, Buist WG, Vereijken PFG: Uitsnijden van varkenskarkassen voor het opstellen van een formule om het vleespercentage met de HGP7 (Hennessy Grading Probe), CGM (Capteur Gras/Maigre-Sydel) en de CSB (Image Meater) te schatten = Lean meat equation for the Hennessy Grading Probe (HGP7), Capteur Gras/Maigre-Sydel (CGM and CSB-Image-Meater (CSB). Wageningen URLivestock Research; 2011.

43.   Centraal Veevoeder Bureau: CVB table pigs. Wageningen, the Netherlands: Centraal Veevoeder Bureau; 2018.

44.   Bird AR, Vuaran M, Brown I, Topping DL: Two high-amylose maize starches with different amounts of resistant starch vary in their effects on fermentation, tissue and digesta mass accretion, and bacterial populations in the large bowel of pigs. *Br J Nutr* 2007, 97:134-144.

45.   Martin LJ, Dumon HJ, Champ MM: Production of short-chain fatty acids from resistant starch in a pig model. *J Sci Food Agric* 1998, 77:71-80.

46.   Heijnen M-LA, Beynen AC: Consumption of retrograded (RS3) but not uncooked (RS2) resistant starch shifts nitrogen excretion from urine to feces in cannulated piglets. *J Nutr* 1997, 127:1828-1832.

47.   Mosenthin R, Sauer W, Henkel H, Ahrens F, De Lange C: Tracer studies of urea kinetics in growing pigs: II. The effect of starch infusion at the distal ileum on urea recycling and bacterial nitrogen excretion. *J Anim Sci* 1992, 70:3467-3472.

48.   Van der Meulen J, Bakker G, Bakker J, De Visser H, Jongbloed A, Everts H: Effect of resistant starch on net portal-drained viscera flux of glucose, volatile fatty acids, urea, and ammonia in growing pigs. *J Anim Sci* 1997, 75:2697-2704.

49.   Jonathan MC, Haenen D, da Silva CS, Bosch G, Schols HA, Gruppen H: Influence of a diet rich in resistant starch on the degradation of non-starch polysaccharides in the large intestine of pigs. *Carbohydr Polym* 2013, 93:232-239.

50.   Wilfart A, Montagne L, Simmins H, Noblet J, Van Milgen J: Digesta transit in different segments of the gastrointestinal tract of pigs as affected by insoluble fibre supplied by wheat bran. *Br J Nutr* 2007, 98:54-62.

51.   Black J, Williams B, Gidley M: Metabolic regulation of feed intake in monogastric mammals. In *Voluntary feed intake in pigs*. Wageningen Academic Publishers, Wageningen, Netherlands; 2009: 189-214

52. Chan HT, Leh CP, Bhat R, Senan C, Williams PA, Karim AA: Molecular structure, rheological and thermal characteristics of ozone-oxidized starch. *Food Chem* 2011, 126:1019-1024.

53. Da Silva CS, Haenen D, Koopmans SJ, Hooiveld GJ, Bosch G, Bolhuis JE, Kemp B, Müller M, Gerrits WJ: Effects of resistant starch on behaviour, satiety-related hormones and metabolites in growing pigs. *animal* 2014, 8:1402-1411.

54. Kidder D, Manners M: The level and distribution of carbohydrases in the small intestine mucosa of pigs from 3 weeks of age to maturity. *Br J Nutr* 1980, 43:141-153.

55. Bolhuis J, Van den Brand H, Staals S, Gerrits W: Effects of pregelatinized vs. native potato starch on intestinal weight and stomach lesions of pigs housed in barren pens or on straw bedding. *Livest Sci* 2007, 109:108-110.

56. So P-W, Yu W-S, Kuo Y-T, Wasserfall C, Goldstone AP, Bell JD, Frost G: Impact of resistant starch on body fat patterning and central appetite regulation. *PLoS One* 2007, 2:e1309.

Chapter 2

Chapter 3

Reduced feed intake, rather than increased energy losses explains variation in growth rates of normal birth weight piglets

Rik J.J. van Erp, Hubert M.J. van Hees, Ruurd T. Zijlstra, Theo A.T.G. van Kempen, Jan Bert van Klinken, Walter J.J. Gerrits

Abstract

Substantial variation in growth rates exists in normal-birth-weight piglets, possibly due to differences in energy efficiency. Within this population, slow growth rates are associated with reduced insulin sensitivity. Slowly digestible starch (SDS) may improve growth efficiency in slowly growing pigs, because it reduces postprandial blood glucose. The aim of this study was to investigate maintenance energy requirements and efficiency of energy used for growth (incremental energy efficiency) of slow- growing or fast- growing piglets (SG-pigs and FG-pigs, respectively) with equal birth weight, that were fed either a SDS or a rapidly digestible-starch (RDS) diet. Sixteen groups of either five 10-wk-old SG-pigs (11.3±1.4 kg) or FG-pigs (15.1±1.7 kg) were housed in climate respiration chambers and fed diets containing 40% RDS or SDS for 2 wk. In week 1, feed was available *ad libitum*. In week 2, feed supply was restricted to 65% of the observed weekly averaged feed intake (kJ·kg body weight $(BW)^{-0.6}·d^{-1}$) in week 1. After week 2, pigs were feed-deprived for 24 h, after which heat production was determined. Energy balances, apparent total tract digestibility (ATTD), and incremental energy efficiencies were calculated and analyzed using a general linear model. Gross energy intake (kJ·kg $BW^{-0.6}·d^{-1}$) was 4% greater (P=0.047) for FG-pigs than for SG-pigs. ATTD of fat was 6 %-units greater (P=0.003) for RDS-fed than SDS-fed pigs. Fasting heat production and incremental energy efficiencies did not differ between pig types or diets. Incremental use of metabolizable energy for fat retention was 2 %-units (P=0.054) greater for RDS-fed than SDS-fed pigs. A lower energy intake rather than greater maintenance requirements or lower energy efficiency explains the slow growth of SG-pigs. Incremental RDS intake increased fat deposition more than SDS, whereas energy efficiency was not affected. Thus, feeding SDS instead of RDS does not improve growth efficiency but may result in slightly leaner animals.

3.1 Introduction

In piglets, a low growth rate is often associated with low birth weight [1] and reduced feed efficiency [2]. In addition, in piglets with equal birth weight, substantial variation exists in growth rates. In addition to genetic, epigenetic and social dominance factors that contribute to this variation, asphyxiation during the birth process is often associated with slow postnatal growth [3, 4]. In human neonates [5], but also in sheep [6], asphyxia has been related to insulin resistance in later life. In a previous study, Paredes, *et al.* [7] selected slow- and fast- growing pigs from a population with normal birth weight that were equal in birth weight but with a BW below the mean - 1 SD or above the mean + 1 SD at 6 wk of age, respectively. This resulted in a BW difference of 4.5 kg at weaning between groups, which together represented a sizeable portion of the piglet population (>20%). They showed that the difference in growth rate could be associated with a 70% difference in insulin sensitivity, which was measured by a hyperglycemic clamp test (1.0 compared with 1.7 mmol glucose · kg $BW^{-0.6}$ · min^{-1}).

Reduced insulin sensitivity elevates postprandial blood glucose levels due to a slow disappearance rate of glucose from the blood, which can inhibit feed intake, stimulate lipogenesis, and consequently, decrease feed efficiency. In humans, it has been shown that slowly digestible starch (SDS) improves management of postprandial hyperglycemia in patients with diabetes [8-11]. A slower rate of starch digestion slows down insulin release [12, 13], which may help pigs with reduced insulin sensitivity to cope with increased postprandial blood glucose concentrations that may improve growth efficiency in SG-pigs with normal birth weight.

Reduced growth efficiency in pigs may be explained by a lower nutrient digestibility [14, 15], a greater maintenance energy requirement [15], a lower efficiency of energy used for growth (i.e., incremental energy efficiency) [16], or a combination of these. Maintenance energy typically reflects energy expenditure for processes that are essential for an organism to remain alive, such as circulation, respiration, thermoregulation, protein turnover, and ion-pumping, whereas incremental energy efficiency represents the heat produced associated with tissue deposition, taking changes in protein and fat deposition into account. We investigated the differences in energy efficiency between SG- and FG-pigs of normal birth weight, populations previously shown to differ in insulin sensitivity [7]. In addition, the effect of the rate of starch digestion on energy efficiency was investigated, because SDS may alleviate the consequences of a reduced insulin sensitivity for growth. We hypothesized that slowly SG-pigs have either an increased maintenance energy expenditure or a lower incremental energy efficiency than FG-pigs. SDS may reduce lipid accretion and hence improve energy efficiency for growth, in particular for SG-pigs.

Chapter 3

3.2 Materials & methods

The experimental protocol was approved by the Animal Care and Use Committee of Wageningen University.

3.2.1 Experimental design

In total, 40 SG-pigs (mean ± SD: 11.3 ± 1.42 kg) and 40 FG-pigs (15.1 ± 1.74 kg) (Hypor Libra x Hypor Maxter) of similar birth weight (1.36 ± 0.13 kg) were selected from 2 weaning batches at the "Swine research Centre" of Nutreco (Boxmeer, Netherlands) following the selection procedure described by Paredes, *et al.* [7]. Per weaning batch, pigs with a normal birth weight (mean birth weight ± 1 SD) were divided into average, slow, and fast growers. From each weaning batch, 30 slow growers (SG-pigs; BW less than the mean – 1 SD) but without clinical signs of disease and 30 fast growers (FG-pigs; BW greater than the mean + 1 SD) were selected at 6 wk of age, and within each performance group assigned to 1 of 2 diets containing either 40% rapidly digestible starch (RDS) or 40% SDS. At 10 wk of age, 20 typical SG-pigs and 20 typical FG-pigs were randomly selected from each batch of 60 pigs and transported to the research facilities of Wageningen University where the effects of pig type and rate of starch digestion were tested in a 2 × 2 factorial design. For pigs from each batch, measurements were performed in 2 blocks. Each block consisted of an adaptation period and an experimental period. Four combinations of pig types and diets were tested per experimental period, resulting in 4 groups of 5 pigs/combination. An attempt was made to divide boars and gilts equally across groups of pigs and treatments. The length of the adaptation differed between blocks (1 compared with 3 wk) due to a limited number of respiration chambers available, resulting in an age difference of approximately ~17 d between blocks (i.e., age groups).

The experimental period was divided in 3 parts: during days 0-6, the pigs were allowed *ad libitum* access to their respective diets; during days 7-14, feed intake of the pigs was restricted to 65% of the realized feed intake in the first week; at day 15, pigs were feed-deprived for 24 h after which fasting heat production (FHP) was determined. Incremental energy efficiencies are affected by the maintenance requirements of a pig, which, in turn, is affected by feed intake level [17]. Hence, the comparison of incremental energy efficiencies is biased by voluntary feed intake. By allowing *ad libitum* feed intake in the first week, we were able to determine values for incremental efficiencies close to the maximum voluntary feed intake. In the second week, feed intake was restricted to 65% of the intake realized in the first week to allow us to measure incremental energy efficiencies within each experimental group of pigs.

3.2.2 Housing

During the adaptation period pigs were housed in 10 pens (1.75 × 2.85 m) with 40% slatted floor. At the start of the experimental period, pigs were housed in 1 of 4 identical climate respiration chambers. Each chamber contained one pen similar in size to the pens used in the adaptation period. The volume of each chamber was reduced from 34.5 m^3 to 27.5 m^3 by inserting airtight material (7 m^3). To ensure that the pigs remained within their thermoneutral zone, temperature was step-wise decreased from 23°C to 20°C during a period of 17 d in the first block of each weaning batch. For the second blocks, which comprised older pigs, temperature was kept constant at 20°C. During the whole experiment, relative humidity was maintained at 65%. Pigs were exposed to 16 h of light (from 06.00h to 22.00h) and 8 h of darkness.

3.2.3 Diets and feeding

Pigs were fed a pelleted diet. Diets were identical except for the starch source used (Table 3.1). The RDS diet contained 37.6% rice starch (Remyline AX-DR, Remy Industries). The SDS diet contained 40% field pea starch (Nastar field pea starch, Cosurca Group). Both starch sources are primarily digested in the small intestines (97.5% compared with 88.9%) [18], but show a clear difference in the rate of starch digestion [12, 19]. The starch inclusion level for the RDS diet was adjusted to compensate for a lower dry matter (DM) content of SDS starch (Table 3.1). Titanium dioxide was added as an indigestible marker at 2 g/kg for determining apparent total tract digestibility (ATTD). Diets were formulated with a net energy of 10.35 MJ/kg and a ratio of standard ileal digestible amino acids to net energy slightly (15%) above the recommendations for piglets in the weight range from 11–25 kg (i.e., 14.4 g standard ileal digestible Lys/kg) [20] to ensure that not protein, but available energy, was limiting the growth rate of the pigs. In the first week of the experiment, feed was available *ad libitum*. In the second week, feed intake was restricted to 65% of the observed weekly average of feed intake/kg $BW^{0.6}$ in the first week. The amount of feed offered in week 2 was adjusted daily on the basis of the pig's weight and expected daily gain, which was based on observed growth in week 1 corrected for a reduction of 65% in growth. In week 2, pigs were fed 2 times/d at 07.30h and 15.30h in a feeding trough with 5 feeding spaces, allowing pigs to eat simultaneously. Feed troughs were closed 30 min after feeding and unconsumed feed was removed and collected. Before the start of the feed-deprivation period, pigs were fed 2 meals, evening and morning, with a fixed amount of 50 g/kg $BW^{0.6}$, which was based on the average feed consumption during week 2 of all pigs in batch 1. Water was available *ad libitum* throughout the experiment. On d 5 of week 2, 2 mg [U-$^{13}C_6$] glucose/kg BW, inserted in a marshmallow, was fed to all pigs inside the respiration chamber 1 h after the evening meal [21]. At this time, the rate of postprandial dietary glucose

absorption reaches its maximum [19], allowing ^{13}C-labeled glucose to trace the metabolic fate of dietary glucose derived from starch. During the feed-deprivation period, [U-^{13}C$_6$] glucose, inserted in a marshmallow, was fed 12 h after the last meal when all dietary glucose is absorbed and blood glucose is restored to the basal concentration [19].

3.2.4 Measurements and analysis

Pigs were weighed weekly and before and after the feed-deprivation period. During weeks 1 and 2, energy and nitrogen balances were measured per group of pigs over a period of 7 d. In both weeks, fecal grab samples were collected for 6 d and stored at 4 °C. Exchange of oxygen, carbon dioxide, and methane was recorded in 6-min intervals. To check the proper functioning of the chambers, a carbon dioxide recovery test was performed before the start of the experiment, according to procedures described by Heetkamp, *et al.* [22]. In the 4 chambers, 98.9%, 99.7%, 100.4%, and 100.4% of the carbon dioxide released was recovered. Physical activity was recorded continuously by one 1 radar device/chamber [22]. Manure was quantitatively collected, homogenized, sampled and stored at -20 °C at the end of each balance period. After each week, grab samples were homogenized, pooled, and stored at -20 °C. Ammonia in exhaust air was captured in water condensed on the heat exchanger, and in 25% sulfuric acid solution; both were collected at the end of each week. Samples of collected manure and grab samples for each balance period were freeze-dried and ground to pass a 1-mm screen (Retsch, Germany). Feed samples were pooled by experimental group after each balance week and ground to pass a 1-mm screen (Retsch ZM200). Dry matter content of feed, manure, fecal grab samples, freeze-dried manure, and freeze-dried grab feces was determined according to the International Organization for Standardization (ISO) 6496 [23]. Gross energy in feed, feed refusals, freeze-dried manure, and freeze-dried grab feces was determined by using bomb calorimetry according to ISO 9831 [24]. Nitrogen in feed, feed refusals, manure, grab feces, water condensed on the heat exchanger, and 25% sulfuric acid solution was determined by using the Kjeldahl method according to ISO 5893 [25]. Titanium was analyzed in feed and freeze dried fecal samples [26]. Analyses were performed in duplicate. On the day of isotope feeding, the concentration of ^{13}CO$_2$ in expired air was measured in 6-min intervals by non-dispersive infrared spectrometry (ABB group, Switzerland) [27].

Table 3.1 Ingredient composition and analyzed chemical composition of experimental diets.

	Diet	
	RDS	**SDS**
Ingredient (g/kg)		
Field pea starch	-	400
Rice starch	376	-
Soybean meal	173	173
Barley	80.0	80.0
Maize	73.2	73.2
Wheat	70.0	70.0
Wheat middlings	40.0	40.0
Soybean protein concentrate	40.0	40.0
Whey powder, sweet	30.7	30.7
Potato protein	30.0	30.0
Water	24.0	-
Soybean oil	15.0	15.0
Monocalcium phosphate	7.40	7.40
L-lysine • HCl	7.00	7.00
Sodium bicarbonate	6.60	6.60
Calcium formate	5.00	5.00
Calcium propionate	5.00	5.00
Premix1	5.00	5.00
DL-methionine	3.30	3.30
L-threonine	2.60	2.60
Titanium dioxide	2.50	2.50
L-valine	2.30	2.30
L-tryptophan	0.90	0.90
Salt	0.80	0.80
Chemical components (g/kg)		
Dry matter	907	904
Crude fat	21.7	23.6
Crude protein	180	180
NDF[2]	64.9	67.3
Starch	474	470
Sugar	39.6	40.5
Titanium	1.78	1.77
Gross energy, MJ/kg	16.6	16.6

[1]Supplied per kg of feed: Sepiolite, 1,5 g; Retinyl acetate, 8,000 IU; Cholecalciferol, 2,000 IU; all-rac-α-Tocopheryl acetate, 30 mg: Menadione nicotinamide bisulfite, 1.5 mg; Thiamin mononitrate 1.0 mg; Riboflavin, 4.0 mg; Calcium-D-Pantothenate, 13 mg; Niacinamide, 20 mg; Pyridoxine hydrochloride, 1.0 mg; Folic acid, 0.3 mg; Cyanocobalamin, 30 μg; Betaine hydrochloride, 66 mg; Iron(II)sulfate 100 mg; Calcium iodate 1 mg; Copper(II)sulfate 160 mg; Manganese(II)oxide 100 mg; Sodium selenite 0.3 mg. [2]Neutral detergent fiber.

Chapter 3

3.2.5 Calculations

Metabolizable energy (ME) was calculated as gross energy (GE) intake minus energy losses via feces and urine and methane. Energy retention (ER) was calculated as ME intake minus heat production, which was calculated with Brouwer's equation without protein coefficients [28]. Nitrogen retention was determined as nitrogen intake minus nitrogen losses via feces and urine and ammonia in exhaust air captured in sulfuric acid solution and in water condensed on the heat exchanger. Protein retention was calculated as nitrogen retention × 6.25 and multiplied by 23.7 kJ/g to calculate energy retained as protein. Energy retained as fat was calculated as ER minus energy retained as protein. Values are expressed as kJ·kg $BW^{-0.6}$·d^{-1} [29].

Incremental digestion coefficients and energy (in)efficiencies are defined as the percentage of energy or nutrients that are digested, deposited, or dissipated per unit increase in feed intake. The relation is assumed to be linear [17, 30-32] and therefore can be calculated following equation 1 and 2, where Δ is the difference between values observed in the *ad libitum* and restricted feeding period.

[1] Incremental ATTD coefficient $= \frac{\Delta \text{ Digested nutrients}}{\Delta \text{ Gross nutrient intake}} \times 100$

[2] Incremental energy or protein efficiency $= \frac{\Delta \text{ Energy or protein retained}}{\Delta \text{ Gross energy or protein intake}} \times 100$

Respiratory quotient (RQ) was calculated as carbon dioxide produced divided by oxygen consumed. Resting metabolic rate (RMR) and heat production related to activity were estimated from total heat production and activity data using penalized b-spline regression procedures [33]. FHP was estimated as the asymptotic RMR during 24 h (including last feeding) of feed deprivation following equation 3, where t is time and α and β are estimated parameters.

[3] $RMR = FHP + \beta e^{-\alpha t^2}$

Net carbohydrate and fatty acid (FA) oxidation were calculated from gas exchange measurements, without protein and methane coefficients [34]. $^{13}CO_2$ recovery was calculated over a period of 24 h after $^{13}CO_2$ supplementation in week 2. $^{13}CO_2$ recovery during the feed-deprivation period was calculated over a period of 12 h after $^{13}CO_2$ supplementation.

3.2.6 Statistics

A group of 5 pigs housed within 1 chamber was considered the experimental unit. Data are reported as least-square means $\pm$ SEMs. Differences were considered significant if $P<0.05$. For all statistical analyses, SAS 9.3 for Windows was used. Normality of model residuals was checked using the Univariate procedure. Data from energy balances, ATTD, heat production related to physical activity, and RQ per balance week and incremental efficiencies, FHP, and $^{13}CO_2$ recovery were analyzed by using a general linear model, as follows,

$$[4]\ Y_{ijklmn} = \mu + T_i + D_j + B_k + A_l + (T{\times}D)_m + e_{ijklmn},$$

where Y_{ijklmn} = dependent variable, μ = overall mean, T_i = type of pig (FG-pigs or SG-pigs), D_j = diet (SDS or RDS), B_k = weaning batch 1 or 2; A_l = block (i.e., age group 1 or 2), $(T{\times}D)_m$ = interaction effect between the type of pig and diet; and e_{ijklmn} = residual error. Diurnal patterns of total heat production, activity heat, RQ, and $^{13}CO_2$ recovery were analyzed by using the same general linear model by hour. Energy balance parameters, heat production, and RQ were adjusted to equal GE intake by including GE intake as a covariate in the statistical model. If the interaction pig type $\times$ diet tended to be significant ($P<0.1$), Tukey pairwise comparisons were used to check which treatment combinations were significantly different.

3.3 Results

3.3.1 General

Throughout the study, pigs remained healthy during the first and second week of each experimental period. One pig was removed at the start of the feed-deprivation period of the second experimental period due to an increase in body temperature. Due to a technical problem with the radar devices, activity measurements performed during the first 3 days of the first week for batch 1 were not reliable and were discarded for calculation of the average daily heat partitioning. Average daily growth of FG-pigs was greater than that of SG-pigs when feed was available *ad libitum* (872 ± 24.7 compared with 718 ± 24.7 g/d; $P=0.007$) and tended to be greater for FG-pigs than SG-pigs when feed intake was restricted (352 ± 12.7 compared with 318 ± 12.7 g/d; $P=0.074$). Growth was not affected by diet and there was no interaction between the type of pig and diet.

3.3.2 Digestibility

When feed was available *ad libitum*, the ATTD of DM and energy tended to be 0.5 %-unit and 0.7 %-unit respectively greater for RDS-fed pigs than for SDS-fed pigs ($P<0.10$; Table 3.2). Fat digestibility was 6.2 %-units greater ($P=0.001$) for RDS-fed pigs than for SDS-fed pigs when feed was available *ad libitum*, and 4.4 %-units greater ($P=0.002$) when feed supply was restricted. When feed supply was restricted, neutral detergent fiber (NDF) degradation was 1.6 %-units greater ($P=0.020$) for RDS-fed pigs than for SDS-fed pigs and tended to be greater ($P=0.092$) for SG-pigs than for FG-pigs.

Table 3.2 Apparent total tract digestibility of dry matter, fat, nitrogen, energy and neutral detergent fiber when feeding a slowly digestible starch or rapidly digestible starch diet in fast growing and slowly growing pigs, when feed was available *ad libitum* or feed supply was restricted. Values are presented as least square means and pooled standard errors of the mean, n=4.

	FG-pigs[2]		SG-pigs[7]			*P*-value		
ATTD[1], %	RDS[4]	SDS[5]	RDS	SDS	SEM[8]	Pig type	Diet	Pig type × Diet
Ad libitum feeding								
Dry matter	90.7	90.3	90.7	90.0	0.27	0.703	0.077	0.642
Fat	68.8	63.7	70.3	63.0	0.90	0.688	0.001	0.250
Nitrogen	85.5	84.6	85.4	84.0	0.70	0.701	0.136	0.728
Energy	89.8	89.3	89.8	88.9	0.34	0.663	0.073	0.613
NDF[3]	61.3	60.3	60.7	59.4	1.46	0.619	0.445	0.909
Restricted feeding								
Dry matter	91.4	91.0	91.3	91.1	0.20	0.961	0.230	0.596
Fat	69.0	64.4	68.5	64.4	1.04	0.835	0.002	0.825
Nitrogen	87.6	87.1	87.4	87.4	1.00	0.929	0.672	0.692
Energy	90.6	90.2	90.5	90.4	0.26	0.977	0.307	0.547
NDF[3]	63.7	61.2	64.5	63.0	0.71	0.092	0.020	0.487

[1]Apparent total tract digestibility. [2]Fast growing pigs. [3]Neutral detergent fiber. [4]Rapid digestible starch. [5]Slowly digestible starch. [6]Slowly growing pigs. [7]Pooled standard error of the mean.

3.3.3 Energy balance

When feed was available *ad libitum*, GE intake (kJ/kg BW$^{0.6}$) was 4.1% greater ($P=0.047$; Table S3.1) for FG-pigs than for SG-pigs, and tended to be 3.5% greater for SDS-fed pigs than for RDS-fed pigs ($P=0.080$). All other energy balance parameters were adjusted to equal GE intake. Energy losses with manure were 27 kJ/kg BW$^{0.6}$ greater for SDS-fed pigs than for RDS-fed pigs ($P=0.005$), resulting in a 1.3% greater ME intake for RDS-fed pigs ($P=0.003$). Heat production was, on average, 40 kJ/kg BW$^{0.6}$ greater for RDS-fed FG-pigs than the other treatments combinations (pig type × diet; $P=0.039$). ER was 45 kJ/kg BW$^{0.6}$ greater for RDS-fed SG-pigs than for RDS-fed FG-pigs (pig type × diet; $P=0.027$). Energy retained as fat tended to be, on average, 51 kJ/kg BW$^{0.6}$ greater for RDS-fed SG-pigs than the other treatments combinations (pig type × diet; $P=0.078$).
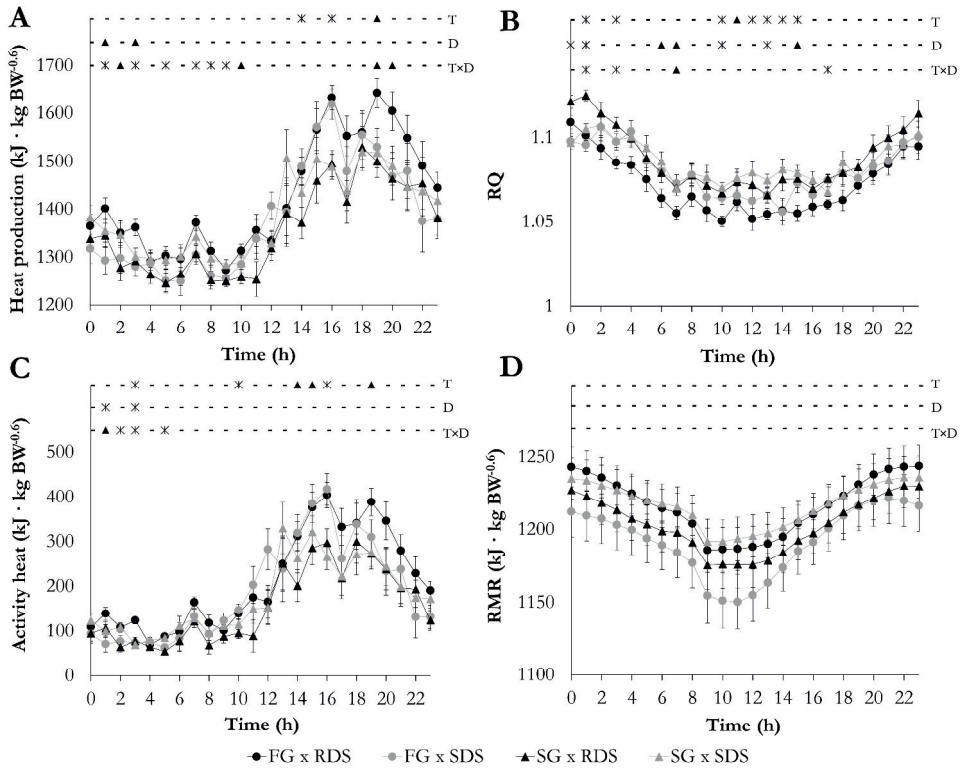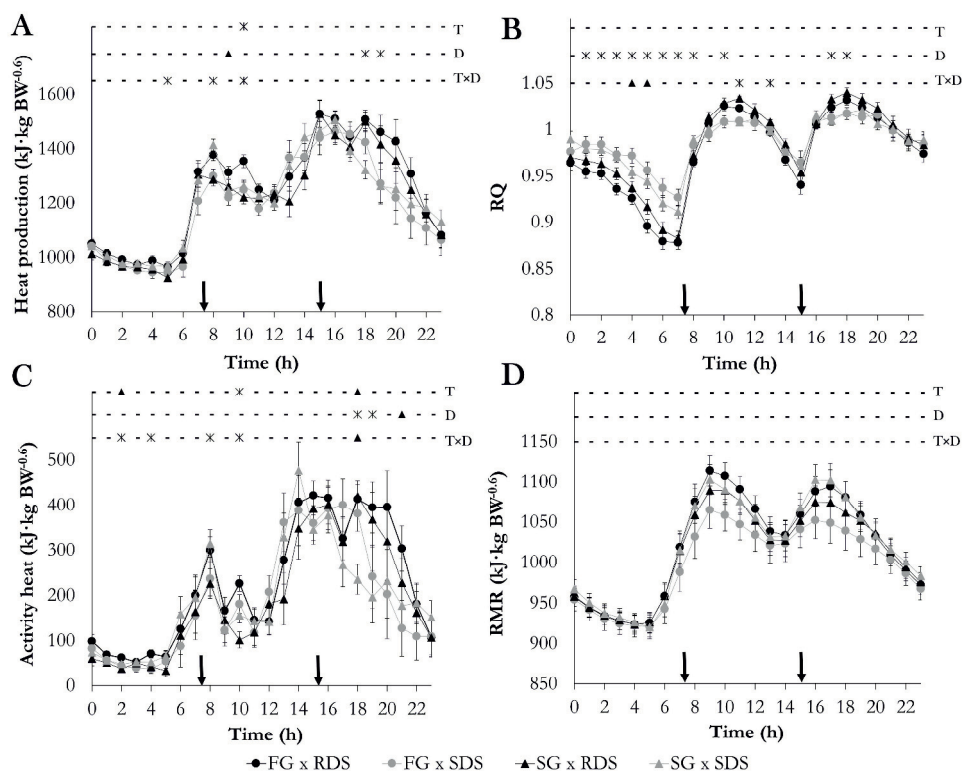
Figure 3.1 Circadian patterns of total heat production (A), RQ (B), activity-related heat production (C), and RMR (D) when feeding an SDS or an RDS diet to FG- and SG-pigs at 10 wk of age, when feed was available *ad libitum*. Values are least-square means ± pooled SEMs, n = 4. Least-square means were adjusted to equal gross energy intake. ✳ represents $P < 0.05$, ▲ represents $0.05 < P < 0.1$, - represents $P > 0.1$. D, diet; FG, fast-growing pig; RDS, rapidly digestible starch; RMR, resting metabolic rate; RQ, respiratory quotient; SDS, slowly digestible starch; SG, slow-growing pig; T, type of pig.

3.3.4 Heat partitioning and substrate oxidation

When feed was available *ad libitum*, total heat production was 70 kJ/kg BW$^{0.6}$ greater for RDS-fed FG-pigs than for RDS-fed SG-pigs (pig type × diet; P=0.052), reaching statistical significance between 01.00h and 10.00h (pig type × diet; P<0.05; Figure 3.1). Activity related heat production was 55 kJ/kg BW$^{0.6}$ greater for RDS-fed FG-pigs than for RDS-fed SG-pigs (pig type × diet; P=0.033; Table S3.2), reaching statistical significance between 02.00h and 06.00h (pig type × diet; P<0.05; Figure 3.1). No differences in activity heat were observed when feed supply was restricted; however, activity was greater for RDS-fed pigs than for SDS-fed pigs between 18.00h and 20.00h (P<0.05), which resulted in a greater total heat production for RDS-fed pigs between these hours (P<0.05; Figure 3.2). When feed was available *ad libitum*, RQ was 0.01 greater (P=0.007) and net FA oxidation was 1.2 g/kg BW$^{0.6}$

Figure 3.2 Circadian patterns of total heat production (A), RQ (B), activity-related heat production (C), and RMR (D) when feeding an SDS or an RDS diet to FG- and SG-pigs at 10 wk of age, when feed supply was restricted. Arrows represent feeding times. Values are least-square means ± pooled SEMs, $n = 4$. Least-square means were adjusted to equal gross energy intake. ∗ represents $P < 0.05$, ▲ represents $0.05 < P < 0.1$, - represents $P > 0.1$. D, diet; FG, fast-growing pig; RDS, rapidly digestible starch; RMR, resting metabolic rate; RQ, respiratory quotient; SDS, slowly digestible starch; SG, slow-growing pig; T, type of pig.

lower (P=0.001) for SG-pigs. For each treatment, RQ exceeded unity continuously, and net FA oxidation remained negative throughout the day (Figure S3.1). With restricted feed intake, RQ was greater in SDS-fed pigs than in RDS-fed pigs before the morning meal, reaching statistical significance between 01.00h and 09.00h, whereas RQ was greater with RDS after both meals (P<0.05). Circadian patterns of net carbohydrate and FA oxidation corresponded with patterns observed for RQ, with a greater net FA oxidation for RDS-fed pigs before the morning meal and greater net carbohydrate oxidation with RDS-fed pigs after the afternoon meal (Figure S3.2). The type of pig or diet did not affect the recoveries of orally supplied [U-^{13}C$_6$] glucose as ^{13}CO$_2$ during both restricted feeding and feed deprivation. Recoveries were 76.2% during restricted feeding (24 h after isotope

supplementation) and 57.5% during feed deprivation (12 h after isotope supplementation; Table S3.2). At 3 h after isotope supplementation, after 15 h of fasting, ^{13}C-recovery was 1.9 %-units greater for SDS-fed pigs than for RDS-fed pigs (P=0.015; Figure S3.3).

3.3.5 Incremental efficiencies

Incremental ATTD coefficients for DM, energy, nitrogen, and NDF were not affected by the type of pig or diet. Incremental ATTD for fat was 9.66 %-units lower for SDS-fed pigs than for RDS-fed pigs (P=0.003), but no differences between pig types were observed (Table 3.3).

Table 3.3 Incremental apparent total tract digestibility coefficients when feeding a slowly digestible starch or rapidly digestible starch diet in fast growing and slowly growing pigs at 10 weeks of age. Values are presented as least square means and pooled standard errors of the mean, n=4.

Incremental ATTD[1] coefficients, %	FG-pigs[2]		SG-pigs[6]			P-value		
	RDS[4]	SDS[5]	RDS	SDS	SEM[7]	Pig type	Diet	Pig type × Diet
Digestible nutrient intake, g·kg BW$^{-0.6}$·d^{-1}								
Dry matter	88.7	88.4	89.0	86.9	0.81	0.504	0.176	0.283
Protein	81.7	80.9	82.0	78.4	1.72	0.539	0.235	0.444
Fat	68.7	62.3	73.0	60.1	2.48	0.682	0.003	0.218
NDF[3]	57.9	58.2	54.9	52.2	3.16	0.188	0.717	0.644
Digestible energy intake, kJ·kg BW$^{-0.6}$·d^{-1}								
Energy	88.2	87.8	88.6	86.5	0.76	0.536	0.122	0.277

[1]Apparent total tract digestibility. [2]Fast growing pigs. [3]Neutral detergent fiber. [4]Rapid digestible starch. [5]Slowly digestible starch. [6]Slowly growing pigs. [7]Pooled standard error of the mean. [8]Incremental digestion coefficients are calculated as percentage of nutrients or energy digested per unit increase in nutrient intake (g·kg BW$^{-0.6}$·d^{-1}) or energy intake (kJ·kg BW$^{-0.6}$·d^{-1}).

Incremental GE and ME efficiency for ER and energy retained as protein were not different for pig types or diets (Table 3.4). Incremental GE efficiency (P=0.065) and ME efficiency (P=0.054) for energy retained as fat were 2.39 %-units and 1.96 %-units greater for RDS-fed pigs than SDS-fed pigs. No effects of pig type or diet were found on incremental GE or ME lost as activity-related heat production or RMR. The effect of incremental GE and ME intakes on total heat production, RMR, and ER was affected by a batch × age group interaction. This effect was primarily caused by a greater incremental heat loss with RMR in the first age group of the second batch. FHP was not different between SG-pigs and FG-pigs (Table 3.4).

Chapter 3

Table 3.4 Incremental responses of energy and protein expenditure to increases in dietary gross energy, metabolizable energy, and protein intake, and fasting heat production and respiratory quotient when feeding a slowly digestible starch or rapidly digestible starch diet, in fast growing and slowly growing pigs at 10 weeks of age. Values are presented as least square means and pooled standard errors of the mean, n=4.

Incremental response, %	FG-pigs[1]		SG-pigs[9]		SEM[10]	P-value		
	RDS[5]	SDS[8]	RDS	SDS		Pig type	Diet	Pig type × Diet
GE[3] intake, kJ·kg BW[-0.6]·d^{-1}								
Feces + urine	8.74	8.74	9.47	11.8	1.50	0.242	0.465	0.465
Methane	0.03	0.09	0.14	0.13	0.07	0.327	0.751	0.677
ME[4]	91.2	91.2	90.4	88.1	1.50	0.225	0.455	0.476
Heat	21.2	21.5	19.4	21.1	0.79	0.195	0.237	0.433
Activity heat	-1.30	-1.66	-3.36	-1.62	1.35	0.474	0.625	0.458
Energy retention	70.1	69.6	71.0	67.0	1.59	0.607	0.206	0.297
As fat	49.1	47.4	50.2	47.2	1.14	0.705	0.065	0.581
As protein	20.9	22.3	20.7	19.9	0.76	0.123	0.778	0.178
ME[4] intake, kJ·kg BW[-0.6]·d^{-1}								
Heat	23.2	23.6	21.6	24.0	0.87	0.502	0.146	0.266
Activity heat	-1.50	-1.86	-3.70	-1.82	1.53	0.497	0.631	0.480
RMR[7]	25.4	24.1	24.7	26.2	1.55	0.652	0.978	0.385
Energy retention	76.8	76.4	78.4	76.0	0.87	0.502	0.146	0.266
As fat	53.9	52.0	55.5	53.4	0.88	0.121	0.054	0.916
As protein	22.9	24.4	22.9	22.6	0.63	0.185	0.392	0.167
Protein intake, g·kg BW[-0.6]·d^{-1}								
Protein retention	81.1	81.3	80.1	74.6	2.50	0.153	0.316	0.280
FHP[2], kJ·kg BW[-0.6]	851	856	838	849	14.3	0.507	0.618	0.844
Fasting RQ[6]	0.76	0.79	0.77	0.78	0.008	0.976	0.055	0.292

[1]Fast growing pigs. [2]Fasting heat production [3]Gross energy. [4]Metabolizable energy. [5]Rapid digestible starch. [6]Respiratory quotient. [7]Resting metbolic rate. [8]Slowly digestible starch. [9]Slowly growing pigs. [10]Pooled standard error of the mean. [11]Incremental responses are calculated as percentage change in energy or protein deposited or dissipated per unit increase in energy or protein intake (kJ or g·kg BW[-0.6]·d^{-1}).

3.4 Discussion

Low growth performance is often explained by a low birth weight caused by intrauterine growth restriction, which is defined as impaired growth of the fetus during pregnancy [1]. However, it has been shown that growth rate with normal birth weight may be reduced as well [16] (i.e., SG-pigs). One of the main reasons for reduced growth is a low feed intake [35, 36]. In our study, GE intake per kilogram BW[0.6] was 4.1% lower for SG-pigs than FG-pigs. In addition, a reduced feed efficiency has been observed in SG-pigs [2]. Reduced feed

efficiency might be explained by a reduced nutrient digestibility; however, this was not corroborated by our findings, because differences in energy (0.2 %-unit), protein (0.3 %-unit), and fat (0.4 %-unit) digestion between pig types were minor and not significant. These results are in line with Jones, *et al.* [15] who found a numerical difference of 0.3% in energy digestibility between pigs that grew fast and pigs that grew slowly after weaning. In the present study, both maintenance energy requirements and incremental energy efficiencies of SG-pigs and FG-pigs were measured to investigate the difference in energy efficiency between pig types. In addition, a gradual release of glucose with SDS [19] may have helped SG-pigs to cope with elevated postprandial glucose levels.

3.4.1 Starch digestion

Field pea starch has a greater fraction of resistant starch (RS) than does rice starch when measured *in vitro* (61.2% vs. 3.1%) [37]. RS escapes enzymatic digestion and becomes a substrate for microbial fermentation, resulting in production of short-chain fatty acids (SCFA) [38-41]. However, the difference in RS between field pea starch and rice starch measured *in vivo* is much smaller [19]. As a result, only a small numerical increase in net portal SCFA appearance was observed in pigs fed a field pea starch diet [19]. Although starch fermentation was not measured directly in this study, and the difference in starch fermentation between diets is expected to be small, greater energy losses with feces and urine and a lower DM, fat, and numerically nitrogen digestibility corroborate that fecal excretion of microbial biomass is increased with SDS. These results are in line with Gerrits, *et al.* [42], who presented a greater fecal output of DM and fat in pigs fed diets containing retrograded maize starch than in those fed pre-gelatinized maize starch. Because there are no indications that starch digestibility alters the digestibility of other nutrients, ~90% of the difference in fecal energy losses between RDS and SDS can be explained by increased fermentation with SDS, assuming that the ratio between protein, fat, and carbohydrates in microbial biomass is equivalent [43]. In addition, microbes may more easily degrade RS compared to other fibrous material. Therefore, RS is preferred as a substrate for microbial fermentation, which could explain the lower NDF degradation for SDS-fed pigs than for RDS-fed pigs [44-46]. Despite an increased level of fermentation with SDS, methane emission was not greater for SDS-fed pigs. These results are in line with earlier studies that also showed that RS fermentation does not induce methane emission [42, 47, 48]. It was suggested that alternative routes of hydrogen excretion must exist, possibly by breath or reductive acetogenesis [42, 49].

3.4.2 Substrate oxidation

RDS initially results in a greater and faster postprandial uptake of glucose than does SDS [12, 50] and, consequently, a greater postprandial excess of glucose. This excess in glucose

is partly stored as fat through *de novo* FA synthesis as is indicated by a greater RQ (exceeding unity) after a meal for RDS-fed pigs than for SDS-fed pigs (Figure 3.2). These results correspond to earlier findings in humans [51, 52] and pigs [42], which all showed a greater increase in postprandial RQ with RDS. In contrast, the decrease in RQ before the morning meal was greater for RDS-fed pigs than for SDS-fed pigs when feed intake was restricted. This postprandial decrease in RQ indicates that the shift from glucose oxidation to fat oxidation due to a decreasing supply of dietary glucose occurs more rapidly with RDS and is also observed after a high-glycemic-index meal in humans [53]. The observations during restricted feeding show that RDS results in a sharp postprandial peak of glucose oxidation followed by the fasting state, whereas SDS attenuates glucose oxidation and increases absorption of SCFAs produced by microbial fermentation, postponing the fasting state. Even after 15 h of feed deprivation, the level of glucose oxidation is still greater in SDS-fed pigs than RDS-fed pigs, which was indicated by a greater ^{13}C-recovery in breath after supplementation of [U-$^{13}C_6$] glucose when animals were feed deprived. After prolonged feed deprivation, glucose oxidation shifts to fat oxidation and *de novo* FA synthesis decreases for both diets, which is indicated by a low RQ (mean: 0.78) after 24 h of fasting.

3.4.3 ER

When feed was available *ad libitum*, the net FA oxidation rates remained negative throughout the day, indicating substantial rates of *de novo* FA synthesis for both pig types. Due to a greater and more continuous uptake of dietary glucose when feed was available *ad libitum*, less postprandial accreted fat was mobilized in the fasting state than with restricted feeding. This may have resulted in a greater fat retention in RDS-fed pigs than in SDS-fed pigs. However, energy retained as fat was only increased in RDS-fed SG-pigs when feed was available *ad libitum*. Greater activity heat in RDS-fed FG-pigs compensated for the difference in fat retention with RDS-fed SG-pigs and resulted in the same amount of energy retained for RDS-fed FG-pigs as SDS-fed FG-pigs and SG-pigs. In contrast to RDS-fed FG-pigs, total heat production of RDS-fed SG-pigs was similar to SDS-fed FG-pigs and SG-pigs. Consequently, a greater amount of energy was retained in RDS-fed SG-pigs than in RDS-fed FG-pigs, which was mainly stored as fat. These results are in accordance with the commonly observed association between fat deposition and insulin sensitivity [54-56].

3.4.4 FHP and incremental efficiencies

A greater energy requirement for maintenance is proposed to be one of the reasons of a reduced postnatal growth rate [16, 35]. However, FHP did not differ between pig types, indicating that maintenance energy requirements were similar. FHP was, on average, slightly greater (848 kJ/kg BW$^{0.6}$) than values of FHP (661 – 774 kJ/kg BW$^{0.6}$) measured in other studies using pigs with a BW ranging from 27 to 88 kg [17, 57, 58]. Similar to our study, pigs

in these other studies were feed deprived for 24 h; however, in contrast to other studies, pigs in our study were group-housed which may have affected the estimate of FHP. Incremental energy efficiencies and incremental digestibility coefficients were not affected by the type of pig, indicating that the reduced GE intake per kilogram $BW^{0.6}$ rather than greater maintenance energy requirements or a reduced incremental energy efficiency is the major driver for slow growth in pigs with normal birth weight. Because nutrient digestibility was similar between pig types, a lower GE intake per kilogram $BW^{0.6}$ with SG-pigs than with FG-pigs may be explained by a slower uptake of glucose by body tissues in SG-pigs, as was found by Paredes, et al. [7]. The values of incremental energy efficiencies (mean: 77%) are somewhat greater compared with values ranging from 67% to 75% measured in other studies [17, 32, 59, 60]. Applying a fixed sequence of feed intakes in time may have led to a slight overestimate of incremental energy efficiencies measured in our study due to a carry-over effect of feeding level in week 1 on FHP in week 2 [57]. The incremental efficiency to store dietary protein as body protein (mean: 79%) is therefore possibly also greater than incremental efficiencies of digestible protein utilization reported by Batterham, et al. [61] (75%) and Bikker, et al. [30] (70%). In addition, this may be explained by genetic improvement, because feed conversion rate has served as a key selection criterion for genetic progress that resulted in leaner animals [62]. We expected a beneficial effect of a slower rate of starch digestion on incremental energy efficiency in SG-pigs. However, incremental GE and ME efficiency for ER did not differ between pig types or diets. RDS-fed pigs did have a greater incremental use of GE and ME for fat retention than did SDS-fed pigs, whereas incremental use of GE and ME for protein retention was not affected by either the type of pig or diet. The incremental ATTD of fat was also greater for RDS-fed pigs than for SDS-fed pigs. However, incremental fecal fat could only explain 15% of the difference in incremental use of GE for fat retention. Therefore, the difference in incremental efficiency of GE for fat retention is likely better explained by greater levels of postprandial fat deposition in RDS-fed pigs rather than by an increase in fecal fat losses. In addition, with increasing feed intake, more substrate is available for fermentation, which might have increased microbial activity and, thus, microbial fat synthesis in SDS-fed pigs. Consequently, more non-dietary fat is excreted, which lowers ATTD fat digestion but not necessarily true fat digestion [63, 64].

3.5 Conclusions

This study provides a better insight in the cause of low growth performance of pigs with similar normal birth weight. According to our results, SG-pigs do not have greater energy requirements for maintenance nor a lower efficiency of energy used for growth than do FG-pigs. Rather, a reduced energy intake (per kilogram $BW^{0.6}$) explains the difference in growth

performance between SG-pigs and FG-pigs. RDS increases postprandial fat accretion through *de novo* FA synthesis, and increasing ME intake caused more additional fat deposition in RDS-fed pigs than in SDS-fed pigs. In contrast to our hypothesis, alleviating the problems of reduced insulin sensitivity by feeding SDS did not improve the incremental energy efficiency in SG-pigs.

References

1. Wu G, Bazer FW, Wallace JM, Spencer TE: Board-invited review: intrauterine growth retardation: implications for the animal sciences. *J Anim Sci* 2006, 84:2316-2337.

2. Douglas SL, Edwards SA, Kyriazakis I: Too late to catch up: A high nutrient specification diet in the grower phase does not improve the performance of low birth weight pigs. *J Anim Sci* 2014, 92:4577-4584.

3. Herpin P, Le Dividich J, Hulin JC, Fillaut M, De Marco F, Bertin R: Effects of the level of asphyxia during delivery on viability at birth and early postnatal vitality of newborn pigs. *J Anim Sci* 1996, 74:2067-2075.

4. Panzardi A, Bernardi ML, Mellagi AP, Bierhals T, Bortolozzo FP, Wentz I: Newborn piglet traits associated with survival and growth performance until weaning. *Prev Vet Med* 2013, 110:206-213.

5. Camm E, Martin-Gronert M, Wright N, Hansell J, Ozanne S, Giussani D: Prenatal hypoxia independent of undernutrition promotes molecular markers of insulin resistance in adult offspring. *The FASEB Journal* 2011, 25:420-427.

6. De Blasio MJ, Gatford KL, Harland ML, Robinson JS, Owens JA: Placental restriction reduces insulin sensitivity and expression of insulin signaling and glucose transporter genes in skeletal muscle, but not liver, in young sheep. *Endocrinology* 2012, 153:2142-2151.

7. Paredes SP, Van Kempen TATG, Verstegen MWA, Van Hees HMJ, den Hartog LA, Dekker R, Lozinska L, Prykhod'ko O, Pierzynowski S, Gerrits WJJ, et al: Glycaemic response of piglets suffering from growth retardation after birth. In *Unveiling causes for growth retardation.* Edited by Paredes SP. Wageningen 2014: 105-128

8. Lehmann U, Robin F: Slowly digestible starch–its structure and health implications: a review. *Trends Food Sci Technol* 2007, 18:346-355.

9. Axelsen M, Arvidsson Lenner R, Lönnroth P, Smith U: Breakfast glycaemic response in patients with type 2 diabetes: effects of bedtime dietary carbohydrates. *Eur J Clin Nutr* 1999, 53:706-710.

10. Seal CJ, Daly ME, Thomas LC, Bal W, Birkett AM, Jeffcoat R, Mathers JC: Postprandial carbohydrate metabolism in healthy subjects and those with type 2 diabetes fed starches with slow and rapid hydrolysis rates determined *in vitro*. *Br J Nutr* 2003, 90:853-864.

11. Ells LJ, Seal CJ, Kettlitz B, Bal W, Mathers JC: Postprandial glycaemic, lipaemic and haemostatic responses to ingestion of rapidly and slowly digested starches in healthy young women. *Br J Nutr* 2005, 94:948-955.

12. Van Kempen TA, Regmi PR, Matte JJ, Zijlstra RT: *In vitro* starch digestion kinetics, corrected for estimated gastric emptying, predict portal glucose appearance in pigs. *J Nutr* 2010, 140:1227-1233.

13. Noah L, Lecannu G, David A, Kozlowski F, Champ M: Digestion of starch and glycaemic response to mixed meals in pigs. *Reprod Nutr Dev* 1999, 39:245-254.

14. Jones CK, Patience JF: Variation in nutrient digestibility and energy intake are key contributors to differences in postweaning growth performance. *J Anim Sci* 2014, 92:2105-2115.

15. Jones C, Patience JF, Gabler NK, Main RG: Both weaning weight and post-weaning growth performance affect nutrient digestibility and energy utilization in pigs. *Animal Industry Report* 2011, 657:78.

16. Paredes SP, Jansman AJM, Verstegen MWA, den Hartog LA, Van Hees HMJ, Bolhuis JE, Van Kempen TATG, Gerrits WJJ: Identifying the limitations for growth in low performing piglets from birth until 10 weeks of age. *Animal* 2014, 8:923-930.

Chapter 3

17. Labussière E, Van Milgen J, de Lange CF, Noblet J: Maintenance energy requirements of growing pigs and calves are influenced by feeding level. *J Nutr* 2011, 141:1855-1861.

18. Fouhse JM, Gänzle MG, Regmi PR, Van Kempen TA, Zijlstra RT: High amylose starch with low *in vitro* digestibility stimulates hindgut fermentation and has a bifidogenic effect in weaned pigs. *J Nutr* 2015, 145:2464-2470.

19. Regmi PR, Metzler-Zebeli BU, Gänzle MG, Van Kempen TATG, Zijlstra RT: Starch with high amylose content and low *in vitro* digestibility increases intestinal nutrient flow and microbial fermentation and selectively promotes bifidobacteria in pigs. *J Nutr* 2011, 141:1273-1280.

20. NRC: *Nutrient requirements of swine*. Washington, D.C., USA: National Academic Press; 2012.

21. Bruininx E, Van den Borne J, Van Heugten E, Van Milgen J, Verstegen M, Gerrits W: Oxidation of dietary stearic, oleic, and linoleic acids in growing pigs follows a biphasic pattern. *J Nutr* 2011, 141:1657-1663.

22. Heetkamp MJW, Alferink SJJ, Zandstra T, Hendriks P, Brand Hvd, Gerrits WJJ: Design of climate respiration chambers, adjustable to the metabolic mass of subjects. In *Indirect calorimetry*. Edited by Gerrits WJJ, Labussière E. Wageningen: Wageningen Academic Publishers; 2015: 35-56

23. ISO 6496: *ISO 6496: Animal feeding stuffs - Determination of moisture and other volatile matter content*. Geneva, Switzerland: International Organization for Standardization; 1999.

24. ISO 9831: *ISO 9831: Animal feeding stuff - Determination of gross calorific value - Bomb calorimetry method*. Geneva, Switzerland: International Organization for Standardization; 1998.

25. ISO 5983: *ISO 5983: Animal feeding stuffs - Determination of N content and calculation of rude protein content - Part 1: Kjeldahl method*. Geneva, Switzerland: International Organization for Standardization; 2005.

26. Myers WD, Ludden PA, Nayigihugu V, Hess BW: A procedure for the preparation and quantitative analysis of samples for titanium dioxide. *J Anim Sci* 2004, 82:179-183.

27. Alferink SJJ, Van den Borne JJGC, Gerrits WJJ, Lammers-Wienhoven SCW, Heetkamp MJW: On-line, continuous determination of 13CO2/12CO2 ratios by non-dispersive infrared absorption in indirect calorimetry facilities. *Publication-European Association for animal production* 2003, 109:465-468.

28. Brouwer E: Report of sub-committee on constants and factors. In *Energy metabolism*. Edited by Blaxter KL: Londen-New York: Academic Press; 1965: 442-443

29. Noblet J, Karege C, Dubois S, Van Milgen J: Metabolic utilization of energy and maintenance requirements in growing pigs: effects of sex and genotype. *J Anim Sci* 1999, 77:1208-1216.

30. Bikker P, Verstegen MWA, Kemp B, Campbell RG: Independent effects of energy and protein intake on protein deposition and utilization in growing pigs In *Protein and lipid accretion in body components of growing pigs: effects of body weight and nutrient intake*. Wageningen 1994: 39-55

31. Campbell R, Taverner M, Curic D: Effect of feeding level and dietary protein content on the growth, body composition and rate of protein deposition in pigs growing from 45 to 90 kg. *Anim Sci* 1984, 38:233-240.

32. Close WH, Berschauer F, Heavens RP: The influence of protein: energy value of the ration and level of feed intake on the energy and nitrogen metabolism of the growing pig: 1. Energy metabolism. *Br J Nutr* 1983, 49:255-269.

33. Van Klinken JB, van den Berg SA, Havekes LM, Van Dijk KW: Estimation of activity related energy expenditure and resting metabolic rate in freely moving mice from indirect calorimetry data. *PLoS One* 2012, 7:e36162.

34. Van den Borne JJGC, Heetkamp MJW, Alferink SJJ, Gerrits WJJ: Moving from a complete energy balance towards substrate oxidation: use of stable isotopes In *Indirect calorimetry*. Edited by Gerrits WJJ, Labussière E. Wageningen: Wageningen Academic Publishers; 2015: 87-113

35. Jones CK, Gabler NK, Main RG, Patience JF: Characterizing growth and carcass composition differences in pigs with varying weaning weights and postweaning performance. *J Anim Sci* 2012, 90:4072-4080.

36. Paredes SP, Jansman AJM, Verstegen MWA, Awati A, Buist W, Quiniou N, Hendriks WH, Gerrits WJJ: Analysis of factors to predict piglet body weight at the end of the nursery phase. *J Anim Sci* 2012, 90:3243-3251.

37. Van Kempen T, Pujol S, Tibble S, Balfagon A, Wiseman J, Varley MA, McOrist S, Kemp B: *In vitro* characterization of starch digestion and its implications for pigs. In *62nd Easter School in the Agricultural and Food Sciences, University of Nottingham, Sutton Bonington Campus, UK, July 2007*. Nottingham University Press; 2007: 515-525.

38. Haenen D, Zhang J, da Silva CS, Bosch G, Van der Meer IM, Van Arkel J, Van den Borne JJ, Gutiérrez OP, Smidt H, Kemp B: A diet high in resistant starch modulates microbiota composition, SCFA concentrations, and gene expression in pig intestine. *J Nutr* 2013, 143:274-283.

39. Yin F, Yin Y, Zhang Z, Xie M, Huang J, Huang R, Li T: Digestion rate of dietary starch affects the systemic circulation of lipid profiles and lipid metabolism-related gene expression in weaned pigs. *Br J Nutr* 2011, 106:369-377.

40. Govers MJAP, Gannon NJ, Dunshea FR, Gibson PR, Muir JG: Wheat bran affects the site of fermentation of resistant starch and luminal indexes related to colon cancer risk: a study in pigs. *Gut* 1999, 45:840-847.

41. Bird AR, Vuaran M, Brown I, Topping DL: Two high-amylose maize starches with different amounts of resistant starch vary in their effects on fermentation, tissue and digesta mass accretion, and bacterial populations in the large bowel of pigs. *Br J Nutr* 2007, 97:134-144.

42. Gerrits WJJ, Bosch MW, Van den Borne JJGC: Quantifying resistant starch using novel, *in vivo* methodology and the energetic utilization of fermented starch in pigs. *J Nutr* 2012, 142:238-244.

43. Storm E, Ørskov ER: The nutritive value of rumen micro-organisms in ruminants: 1. Large-scale isolation and chemical composition of rumen micro-organisms. *Br J Nutr* 1983, 50:463-470.

44. Martínez-Puig D, Castillo M, Nofrarias M, Creus E, Pérez JF: Long-term effects on the digestive tract of feeding large amounts of resistant starch: A study in pigs. *J Sci Food Agric* 2007, 87:1991-1999.

45. Jonathan MC, Haenen D, da Silva CS, Bosch G, Schols HA, Gruppen H: Influence of a diet rich in resistant starch on the degradation of non-starch polysaccharides in the large intestine of pigs. *Carbohydr Polym* 2013, 93:232-239.

46. de Vries S, Gerrits WJJ, Kabel MA, Vasanthan T, Zijlstra RT: β-Glucans and resistant starch alter the fermentation of recalcitrant fibers in growing pigs. *PLoS One* 2016, 11:e0167624.

47. Bolhuis JE, Van den Brand H, Staals STM, Zandstra T, Alferink SJJ, Heetkamp MJW, Gerrits WJJ: Effects of fermentable starch and straw-enriched housing on energy partitioning of growing pigs. *Animal* 2008, 2:1028-1036.

48. Schrama JW, Bakker GC: Changes in energy metabolism in relation to physical activity due to fermentable carbohydrates in group-housed growing pigs. *J Anim Sci* 1999, 77:3274-3280.

Chapter 3

49. Graeve KG, Grivet JP, Durand M, Beaumatin P, Cordelet C, Hannequart G, Demeyer D: Competition between reductive acetogenesis and methanogenesis in the pig large-intestinal flora. *J Appl Microbiol* 1994, 76:55-61.

50. Frost G, Keogh B, Smith D, Akinsanya K, Leeds A: Preliminary report The effect of low-glycemic carbohydrate on insulin and glucose response *in vivo* and *in vitro* in patients with coronary heart disease. *Metabolism* 1996, 45:669-672.

51. Sparti A, Milon H, Di Vetta V, Schneiter P, Tappy L, Jéquier E, Schutz Y: Effect of diets high or low in unavailable and slowly digestible carbohydrates on the pattern of 24-h substrate oxidation and feelings of hunger in humans. *Am J Clin Nutr* 2000, 72:1461-1468.

52. Tagliabue A, Raben A, Heijnen ML, Deurenberg P, Pasquali E, Astrup A: The effect of raw potato starch on energy expenditure and substrate oxidation. *Am J Clin Nutr* 1995, 61:1070-1075.

53. Ritz P, Krempf M, Cloarec D, Champ M, Charbonnel B: Comparative continuous-indirect-calorimetry study of two carbohydrates with different glycemic indices. *Am J Clin Nutr* 1991, 54:855-859.

54. Jung UJ, Choi M-S: Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. *Int J Mol Sci* 2014, 15:6184-6223.

55. Reaven GM: Role of insulin resistance in human disease. *Diabetes* 1988, 37:1595-1607.

56. Kahn SE, Hull RL, Utzschneider KM: Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 2006, 444:840.

57. de Lange K, Van Milgen J, Noblet J, Dubois S, Birkett S: Previous feeding level influences plateau heat production following a 24 h fast in growing pigs. *Br J Nutr* 2006, 95:1082-1087.

58. Noblet J, Van Milgen J, Carré B, Dimon P, Dubois S, Rademacher M, Van Cauwenberghe S: Effect of body weight and dietary crude protein on energy utilisation in growing pigs and broilers. *Publication-European Association for animal production* 2003, 109:205-208.

59. Verstegen MWA, Close WH, Start IB, Mount LE: The effects of environmental temperature and plane of nutrition on heat loss, energy retention and deposition of protein and fat in groups of growing pigs. *Br J Nutr* 1973, 30:21-35.

60. Holmes CW, Carr JR, Pearson G: Some aspects of the energy and nitrogen metabolism of boars, gilts and barrows given diets containing different concentrations of protein. *Anim Sci* 1980, 31:279-289.

61. Batterham ES, Andersen LM, Baigent DR, White E: Utilization of ileal digestible amino acids by growing pigs: effect of dietary lysine concentration on efficiency of lysine retention. *Br J Nutr* 1990, 64:81-94.

62. Knap PW, Wang L: Pig breeding for improved feed efficiency. In *Feed efficiency in swine.* Edited by Patience JF2012: 167-181

63. Kil DY, Sauber TE, Jones DB, Stein HH: Effect of the form of dietary fat and the concentration of dietary neutral detergent fiber on ileal and total tract endogenous losses and apparent and true digestibility of fat by growing pigs. *J Anim Sci* 2010, 88:2959-2967.

64. Jørgensen H, Fernández JA: Chemical composition and energy value of different fat sources for growing pigs. *Acta Agriculturae Scandinavica, Section A-Animal Science* 2000, 50:129-136.

Supplementary Data

Table S3.1 Energy balance parameters when feeding a slowly digestible starch or rapidly digestible starch diet in fast growing and slowly growing pigs at 10 weeks of age, when feed was available *ad libitum* or feed supply was restricted. Values are presented as least square means and pooled standard errors of the mean, n=4.

	FG-pigs[1]		SG-pigs[6]		SEM[7]	*P*-value		
	RDS[4]	SDS[5]	RDS	SDS		Pig type	Diet	Pig type × Diet
Ad libitum feeding (kJ · kg BW$^{-0.6}$ · d^{-1})								
GE[2] intake	2600	2742	2549	2585	44.6	0.047	0.080	0.271
Variables adjusted for equal GE intake								
Feces + urine	235	261	238	266	6.39	0.574	0.005	0.805
Methane	10.3	9.2	11.7	11.5	1.07	0.166	0.564	0.673
ME[3] intake	2374	2349	2370	2341	5.69	0.373	0.003	0.722
Heat	1421^a	1385^b	1372^b	1385^b	10.4	0.070	0.323	0.040
Energy retention	953^a	964^{ab}	998^b	957^a	10.1	0.141	0.209	0.027
As protein	490	503	482	486	6.17	0.116	0.228	0.478
As fat	463^a	462^a	516^b	471^a	11.4	0.045	0.094	0.078
Restricted feeding (kJ · kg BW$^{-0.6}$ · d^{-1})								
GE intake	1747	1843	1720	1748	34.2	0.104	0.099	0.349
Variables adjusted for equal GE intake								
Feces + urine	163	180	159	168	8.28	0.366	0.165	0.586
Methane	9.9	9.2	10.2	10.1	0.53	0.288	0.462	0.520
ME intake	1592	1575	1592	1587	8.29	0.402	0.178	0.615
Heat	1241	1188	1208	1203	13.7	0.555	0.079	0.101
Energy retention	352	387	388	384	18.8	0.426	0.446	0.301
As protein	312	304	309	318	5.79	0.349	0.944	0.164
As fat	40.2	84.1	79.3	66.2	16.1	0.551	0.389	0.100

[1]Fast growing pigs. [2]Gross energy. [3]Metabolizable energy. [4]Rapid digestible starch. [5]Slowly digestible starch. [6]Slowly growing pigs. [7]Pooled standard error of the mean. [8]Labeled means in a row without a common letter differ, P < 0.05.

Chapter 3

Table S3.2 Heat partitioning, RQ, substrate oxidation, and $^{13}CO_2$ recovery when feeding a slowly digestible starch or rapidly digestible starch diet in fast growing and slowly growing pigs at 10 weeks of age, when feed was available *ad libitum* and feed supply was restricted. Values are presented as least square means and pooled standard errors of the mean, n=4.

	FG-pigs[1]		SG-pigs[6]		SEM[7]	P-value		
	RDS[3]	SDS[5]	RDS	SDS		Pig type	Diet	Pig type × Diet
Ad libitum feeding								
Total heat production, kJ · kg BW$^{-0.6}$ · d^{-1}	1427[a]	1382[ab]	1357[b]	1390[ab]	18.1	0.152	0.759	0.052
Activity heat, kJ · kg BW$^{-0.6}$ · d^{-1}	210[a]	188[ab]	155[c]	173[bc]	8.39	0.004	0.842	0.033
Resting metabolic rate, kJ · kg BW$^{-0.6}$ · d^{-1}	1217	1194	1202	1217	13.9	0.791	0.779	0.196
RQ[4]	1.072[a]	1.081[ab]	1.088[b]	1.086[b]	0.003	0.007	0.290	0.083
Carbohydrate oxidation, g · d^{-1}	98.2	96.1	98.4	98.7	0.75	0.294	0.117	0.405
Fat oxidation, g · d^{-1}	-7.66[a]	-7.73[ab]	-8.98[b]	-8.83[b]	0.20	0.001	0.836	0.085
Restricted feeding								
Total heat production, kJ · kg BW$^{-0.6}$ · d^{-1}	1242[a]	1186[b]	1203[ab]	1209[ab]	15.9	0.637	0.168	0.070
Activity heat, kJ · kg BW$^{-0.6}$ · d^{-1}	222	187	189	189	12.8	0.280	0.217	0.189
Resting metabolic rate, kJ · kg BW$^{-0.6}$ · d^{-1}	1020	999	1014	1020	14.9	0.653	0.655	0.355
RQ	0.98	0.99	0.98	0.99	<0.01	0.390	0.135	0.171
Carbohydrate oxidation, g · kg BW$^{-0.6}$ · d^{-1}	64.7	63.3	64.8	64.2	0.82	0.596	0.291	0.616
Fat oxidation, g · kg BW$^{-0.6}$ · d^{-1}	2.59	1.88	1.74	1.87	0.35	0.271	0.451	0.239
[U-$^{13}C_6$] glucose recovery as $^{13}CO_2$								
Restricted feeding, %	78.0	73.3	79.6	73.4	3.29	0.740	0.145	0.902
Fasting, %	56.2	59.2	56.5	58.2	3.34	0.920	0.494	0.852

[1]Fast growing pigs. [2]Rapid digestible starch. [3]Respiratory quotient. [4]Slowly digestible starch. [5]Slowly growing pigs. [6]Pooled standard error of the mean. [7]Least square means except for $^{13}CO_2$ recoveries are adjusted to equal gross energy intake. [8]Labeled means in a row without a common letter differ, P < 0.05.
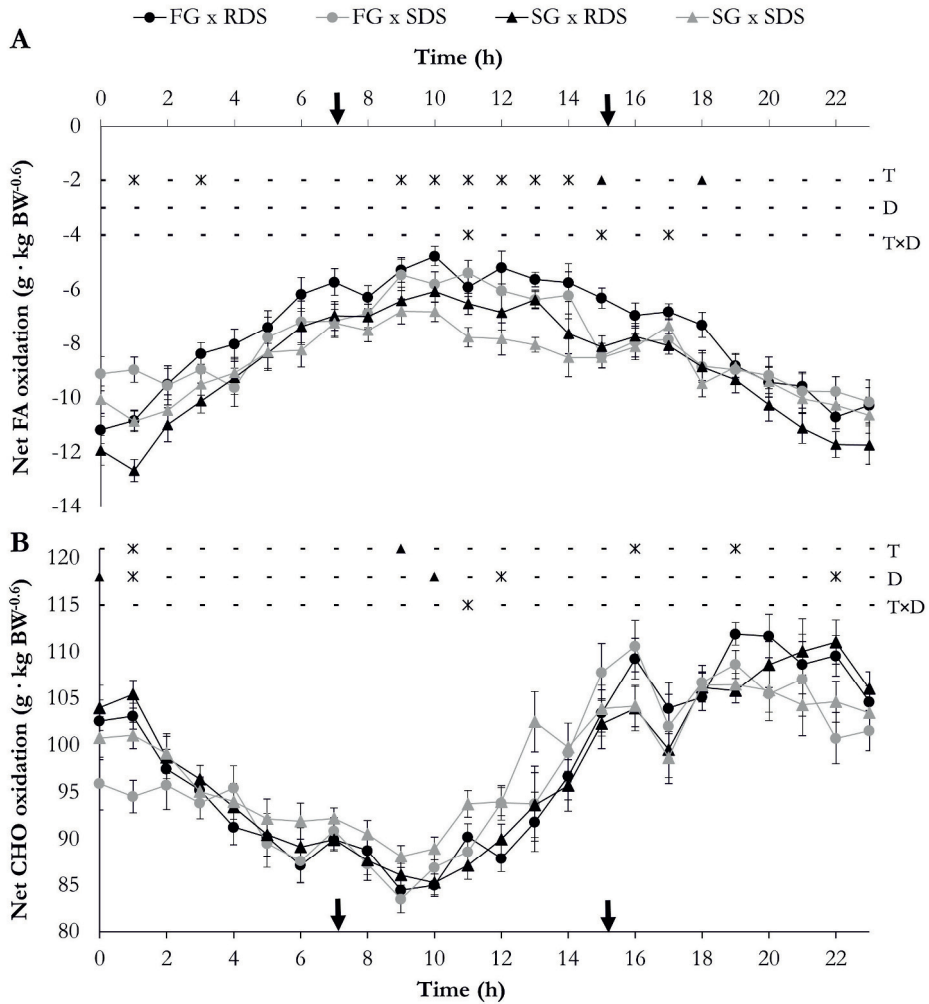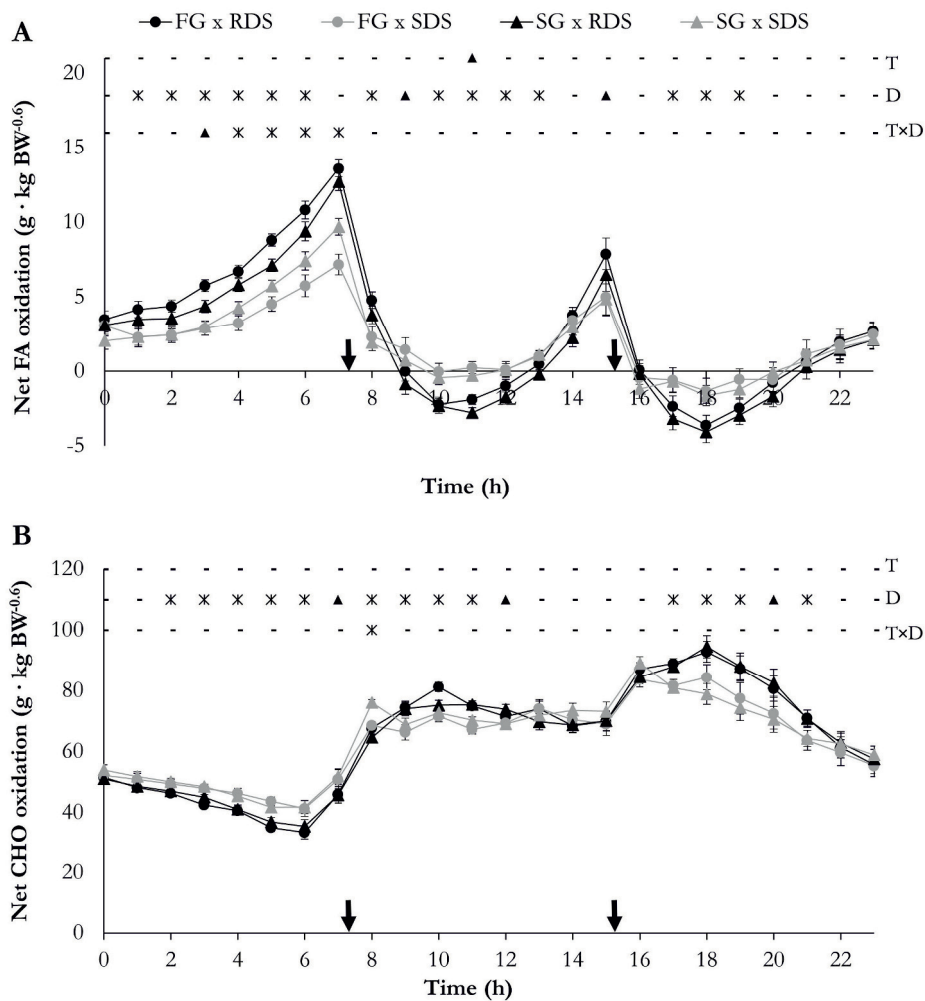
Figure S3.1 LS means of circadian patterns of net fatty acid (panel A) and carbohydrate oxidation (panel B) when feeding a slowly digestible starch (SDS) or rapidly digestible starch (RDS) diet to fast growing (FG) and slowly growing (SG) pigs at 10 weeks of age, when feed was available *ad libitum*. Values are presented as least square means and standard error of the mean, n=4; Least square means are adjusted to equal gross energy intake; ✱represents $P < 0.05$, ▲ represents $0.05 < P < 0.1$, - represents $P > 0.1$; CHO = Carbohydrate; FA = Fatty Acids; D = Diet; T = Type of pig.

Figure S3.2 Circadian patterns of net fatty acid (panel A) and carbohydrate oxidation (panel B) when feeding a slowly digestible starch (SDS) or rapidly digestible starch (RDS) diet to fast growing (FG) and slowly growing (SG) pigs at 10 weeks of age, when feed supply was restricted. Arrows represent feeding times. Values are presented as least square means and pooled standard errors of the mean, n=4; Least square means are adjusted to equal gross energy intake; ✳ represents $P < 0.05$, ▲ represents $0.05 < P < 0.1$, - represents $P > 0.1$; CHO = Carbohydrate; FA = Fatty Acids; D = Diet; T = Type of pig.
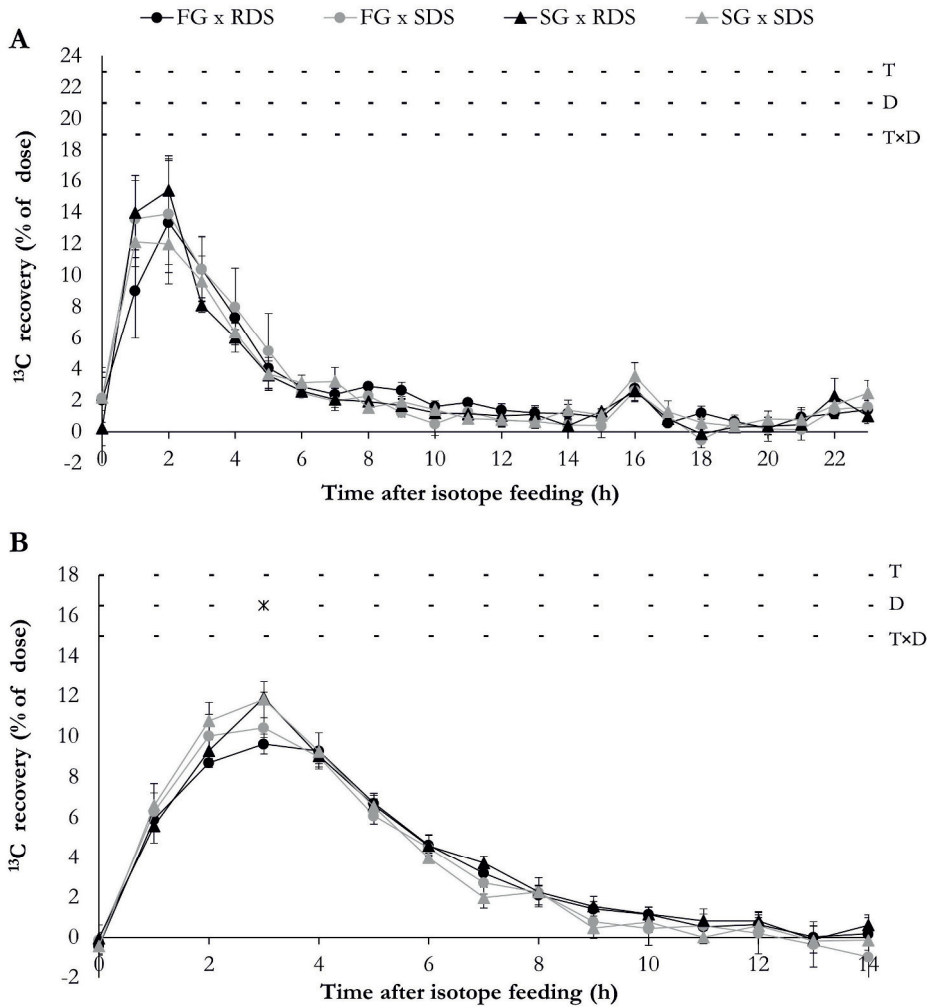
Figure S3.3 ^{13}C recovery of [U-^{13}C$_6$] glucose fed 1 hour after the evening meal when feed supply was restricted (Panel A), and fed 12 h after the last meal during the fasting period (panel B), when feeding a slowly digestible starch (SDS) or rapidly digestible starch (RDS) diet to fast growing (FG) and slowly growing (SG) pigs at 10 weeks of age. Values are presented as least square means and standard error of the mean, n=4; ✳represents $P < 0.05$, ▲ represents $0.05 < P < 0.1$, - represents $P > 0.1$; D = Diet; T = Type of pig.

Chapter 3

Chapter 4

Quantification of ileal and total tract starch fermentation in pigs fed resistant starch

Rik J.J. van Erp, Sonja de Vries, Bart van der Hee, Hauke Smidt, Theo A.T.G. van Kempen, Walter J.J. Gerrits

Abstract

Fermentation of starch in the upper gastrointestinal tract is often ignored, but has been shown to occur. Neglecting starch fermentation may result in an overestimation of ileal starch digestion by host-enzymes when based on starch disappearance. A method based on the contrast in natural ^{13}C-enrichment between starch and non-starch dietary components was developed to quantify total tract fermentation of starch. Using this method, we aimed to quantify the effect of RS on bacterial biomass formation in the small and large intestine. In addition, we evaluated the effects on microbial biomass composition. In this study, twenty male pigs (34.9±1.36 kg) were fed diets containing either 50% waxy maize starch (low in RS; LRS) or high-amylose maize starch (high in RS; HRS), with titanium dioxide as indigestible marker. After 14 days of adaptation to the diets, pigs were fed hourly, dissected, and digesta were collected from the ileum and rectum. Ileal and total tract starch disappearance was measured. Flows of nutrients, DNA, and 16S rRNA gene copies, were calculated and microbial biomass composition was analyzed. Ileal and total tract starch fermentation were determined using the ^{13}C-method. Colonic starch fermentation was calculated as total tract fermentation minus ileal starch disappearance. Total tract starch fermentation was 18 %-units greater in HRS-fed pigs than LRS-fed pigs ($P<0.001$). Colonic starch disappearance was 24 %-units greater in LRS-fed than HRS-fed pigs ($P<0.001$), implying that ileal starch fermentation was 6 %-units greater in LRS-fed pigs than HRS-fed pigs ($P=0.046$). Microbial biomass composition in rectal digesta, but not in ileal digesta, slightly differed between diets, as shown by a shift in weighted unifrac distance and by a greater phylogenetic diversity in LRS-fed pigs than HRS-fed pigs (24.8 vs. 19.2; $P=0.001$). The method used to estimate total tract starch fermentation was reasonably close to colonic starch disappearance, but largely overestimated ileal starch fermentation. This was corroborated by an increase in the rectal DNA flow, which was representative for microbial DNA, coinciding with an increase in the flow of starch-derived C incorporated in microbial biomass. No such relation was observed at the ileum. Our results suggest that ileal starch digestion in pigs is overestimated with 1-7 % when based on ileal starch disappearance, particularly in pigs fed high digestible starch diets.

4.1 Introduction

Starch, disappearing from the small intestine is commonly assumed to be digested by host-enzymes. Starch that remains undigested, so-called resistant starch (RS), flows into the large intestine where it serves as a substrate for microbial fermentation. Although microbial fermentation for the greater part takes place in the large intestine of pigs, microbial activity in the upper gastrointestinal tract has been shown to exist [1-3] suggesting that fermentation already occurs in the upper gastrointestinal tract. Hitherto, the importance of starch fermentation in the upper gastrointestinal tract for the pig has not been established.

RS fermentation is suggested to be beneficial for maintenance of gut health and reduction of risk factors for intestinal problems [4]. In addition, RS may act as a prebiotic as it promotes selective proliferation of commensal bacteria in the large intestine [5-7]. Changes in microbial biomass composition in response to RS intake were shown to be less explicit in ileal digesta [5, 8], but are not as comprehensively investigated than in the large intestine. In addition, methods to analyze starch digestion or glucose yield from starch commonly ignore starch fermentation in the upper gastrointestinal tract. E.g., ileal starch disappearance is measured as starch digestion by host-enzymes and starch fermentation combined, and the difference between postprandial portal glucose appearance and ileal starch disappearance is explained by both microbial starch fermentation and intestinal glucose utilization [9-12]. Neglecting ileal starch fermentation may result in an overestimation of ileal starch digestion by host-enzymes. Gerrits*, et al.* [13] developed a method to quantify total tract starch fermentation. This method is based on the positive difference in natural ^{13}C-enrichment between starch (highly enriched) and non-starch diet (poorly enriched) components, thereby assuming that an increase in ^{13}C-enrichment of the non-starch fraction of digesta is explained by incorporation of ^{13}C-enriched carbon (C) from fermented starch into microbial biomass. This method, described for total tract fermentation of starch, has not yet been applied for small intestinal fermentation.

The magnitude of the effect of starch fermentation on current estimates of starch digestion is yet unknown. In this study, we quantified both ileal and total tract starch fermentation using the method described by Gerrits*, et al.* [13] and investigated the effect of RS on microbial biomass formation and its composition.

4.2 Materials & Methods

The experimental protocol was approved by the Animal Care and Use Committee of Utrecht University, Utrecht, The Netherlands; the experiment was performed at the experimental facilities of Nutreco NV, Sint Anthonis, The Netherlands.

Twenty male pigs (34.9 ±1.36 kg; Hypor Libra x Hypor Maxter, Hendrix Genetics, Boxmeer, the Netherlands) were selected at 11 weeks of age and housed individually in pens

of 2.5m x 0.95m with 40% slatted floors, during a period of 16 d. Pigs were assigned to one of two diets (Table 4.1); low-RS (LRS), containing 50% of waxy maize starch or high-RS (HRS), containing 50% of high amylose maize starch (Roquette, Lestrem, FR). Pigs were exposed to 16 h of light (day 0-14, from 06.00h to 22.00h; day 14-15, from 05.00h to 21.00h) and 8 h of darkness. Temperature was controlled at 23 °C. Water was available *ad libitum* during the entire study period. Pigs were weighed at the start of the experiment, at the first day of the second week, and before dissection.

Table 4.1 Ingredient composition and analyzed chemical composition of experimental diets.

	Diets	
	LRS[3]	**HRS**[4]
Ingredient (g/kg)		
Waxy maize	500.0	-
High amylose maize	-	497.0
Rapeseed meal	150.0	150.0
Sunflower seed meal	150.0	150.0
Wheat gluten meal	94.5	94.5
Water[1]	25.0	28.0
Molasses, cane	25.0	25.0
Palm oil	21.6	21.6
Premix[2]	12.5	12.5
Calcium Carbonate	8.1	8.1
L-Lysine	5.4	5.4
Sodium Bicarbonate	4.3	4.3
Mono-calcium phosphate	2.7	2.7
Choline Chloride	0.1	0.1
L-Threonine	0.6	0.6
L-Tryptophan	0.1	0.1
Phytase	0.1	0.1
Analyzed chemical composition (g/kg)		
Dry matter	886	873
Crude protein (N × 6.25)	179	181
Starch	420	430
Gross energy, MJ/kg	17.7	17.7

[1]Water was included to compensate for a lower dry matter content of high-amylose maize starch. [2]Supplied per kg of feed: Citric acid 111 mg; propyl gallate 69 mg; butyl hydroxy-toluene 151 mg; sepiolite 158 g; vitamin A 8,000 IU; vitamin D3 1600 IU; vitamin E (all-rac-alpha-tocoopheryl acetate) 7.5 IU; vitamin K3 (menadione nicotinamide bisulphate) 160 mg; vitamin B1 (thiamine mononitrate) 80 mg; vitamin B2 (riboflavin) 400 mg; calcium-D-pantothenate 1,3 g; choline chloride 12 g; niacin 1,6 g; vitamin B6 (pyridoxine hydrochloride) 120 mg; folic acid 120 mg; vitamin B12 (cyanocobalamin) 1.6 mg; biotin 12 mg; betaine-hydrochloride 7.9 mg; iron(II) sulphate 8 g; calcium iodate 80 mg; cupper(II) sulphate 12 g; manganese(II) oxide 2.4 g; zinc oxide 8 g; sodium selenite 24 mg. [3]Low resistant starch. [4]High resistant starch.

4.2.1 Experimental design and measurements

In the first two days of the experiment, pigs were gradually switched from a commercial diet (CP 172 g/kg, Net Energy (NE) 9.17 MJ/kg; ABZ, Leusden, Netherlands) to one of the experimental diets. In both experimental diets, titanium dioxide (TiO_2; 3 g/kg) was added as indigestible marker. Feeding level was set at 2.3 times the energy requirements for maintenance (750 kJ of NE/kg$^{0.6}$; [14]). Feed allowance was adjusted daily, based on body weight and an expected daily gain of 600 g/d. Pigs were fed twice a day until day 13, after which daily meal frequency was increased to six meals that were equally distributed between 05.30h and 20.30h. At day 16, pigs were hourly fed with meals that equaled 1/12th of the daily portion. During this period, lights remained on. After a minimum of six meals, pigs were sedated sequentially (one hour after the last meal) by an intramuscular injection of Zoletil® 100 (0.06 ml/kg BW) and an intravenous injection of Euthasol® 20% (24 mg/kg BW) in the ear, after which they were euthanized by exsanguination via the carotid artery. The sequence in which pigs were dissected was blocked to account for the difference in number of meals pigs received before dissection. Each block consisted of two HRS-fed and two LRS-fed pigs. After euthanasia, the abdominal cavity was opened. Surgical clamps were placed at the start and end of the small intestine, and end of the large intestine after which the gastrointestinal tract was removed from the animal. The terminal 1.5 m of the small intestine was considered to represent the ileum. The digesta in both the ileum and rectum were gently squeezed out, collected quantitatively, and stored at -20ºC.

4.2.2 Analytical procedures

Nutrient content and ^{13}C-enrichment

Prior to chemical analyses, digesta samples from each segment were freeze-dried and ground to pass a 1 mm screen. Diets and collected digesta were analyzed for contents of dry matter (DM) [15], nitrogen (N) and C [16], titanium [17], and starch [18]. Digesta samples were analyzed for starch without prior removal of soluble sugars. In starch sources, and in ball-milled non-starch diet ingredients, complete diets, and freeze-dried digesta, ^{13}C-enrichment was analyzed by combustion isotope ratio MS by using a Delta V Advantage isotope-ratio mass spectrometer (Thermo Scientific, Waltham, MA). All analyses were carried out in duplicate, unless indicated otherwise.

DNA extraction procedure

DNA was extracted from freeze-dried ileal and rectal digesta samples. In total, 200 mg per sample was added to a bead-beating tube containing 700 µl Stool Transport and Recovery (STAR)-solution, 0.5 g of 0.1 mm zirconia beads, and five 2.5 mm glass beads. Samples were homogenized by repeated bead-beating (5.5 ms, 3 x 1 min, FastPrep) at room temperature

Chapter 4

(RT) and subsequently incubated at 95 °C for 15 min, while shaking at 300 rpm. After incubation, samples were pelleted by centrifugation at 4 °C (5 min, 15000 x g) and supernatant was transferred to new 1.5 ml Eppendorf tubes on ice. Previous steps were repeated using the pellet and 300 µl STAR-solution, and both resulting supernatants were pooled. Per sample, 250 µl supernatant was used for DNA purification using an automated extraction instrument (Tissue LEV Total RNA purification kit cat. AS1220, Maxwell 16, Promega) and eluted in 50 µl DNAse/RNAse-free water. Total DNA concentrations (mg/g DM digesta) were measured using a DS11 spectrophotometer (DeNovix, Wilmington, DE, USA). Due to limited sample material one observation (treatment HRS) of the ileum was missing.

Total bacterial load estimation by real time qPCR

For bacterial load calculations, DNA was diluted to a final concentration of 1.5 ng/µl. As standard, 10-fold serial dilutions were made of a 1:1 mixture of *Enterotoxigenic Escherichia coli* (ETEC) and *Lactobacillus plantarum* WCSF1 (LbP, from $1 \times 10^8 - 1 \times 10^2$ CFU, Figure S1) and used for calculations in every PCR run. Amplification of the 16S ribosomal RNA (rRNA) gene was performed using Bact1369FW – CGGTGAATACGTTCYCGG and Bact1492RV – GGWTACCTTGTTACGACTT primers [19]. Master mix contained per reaction 7 µl 2x SYBR green (Promega), 0.28 µl FW-primer (10 µM), 0.28 RV-primer (10 µM), 3.64 µl RNAse- and DNAse-free water, and 2.8 µl template DNA (total 4.2 ng DNA). PCR cycles were 94 °C denaturing for 10 minutes, and 34 cycles of 94 °C denaturing (20 seconds), 60 °C annealing (30 seconds), 72 °C elongation (30 seconds), and further extension at 72 °C for 30 seconds. A melt curve was recorded starting from 60 °C with 0.5 °C increments to 95 °C on a Rotor-gene Q2 (Qiagen). PCR was performed in triplicates per sample, and total concentrations were calculated using the standard curve method (Rotor-Gene Q-series software v2.3.1). Total bacterial loads were calculated based on concentrations from initial DNA extraction procedures, calculated per µl sample, and deduced for final concentrations of 16S rRNA gene copies per g freeze-dried digesta.

Library preparation for amplicon sequencing

PCR amplification of the V5-6 region of the 16S rRNA gene was performed in duplicate. Preparation of the master mix contained per reaction 10 µl 5x HF buffer, 1 µl dNTPs (10 mM each dNTP), 0.5 µl Phusion hot start II DNA Polymerase (2U/µl), 36.5 µl RNAse-DNAse-free water, 1 µl uniquely barcoded primers (BSF784FW - RGGATTAGATACCC and 1064RV - CGACRRCCATGCANCACCT), 10 µM each [20], and 1 µl containing 20 ng template DNA. Cycling conditions were 30 seconds denaturation at 98 °C, and 25 cycles of denaturation at 98 °C (10 seconds), annealing at 50 °C (10 seconds), elongation at 72 °C (10 seconds), and a final extension at 72 °C (7 minutes). PCR products were verified on 1% agarose gel (100V, 20 minutes), and purified with MagBio beads following manufacturer's

procedures (MagBio Genomics Inc, Gaithersburg, MD, USA). Total concentrations of DNA were measured on Qubit using the dsDNA Broad-range assay (Invitrogen). Equimolar mixtures of the complete library were pooled (200 ng/sample), concentrated using MagBio beads (final concentration of 200 ng/μl), and libraries were sequenced on a Illumina HiSeq 2000 platform (150 bp, paired-end at GATC Biotech, Konstanz, Germany; now part of Eurofins Genomics Germany GmbH). Due to limited sample material one observation (treatment HRS) of the ileum was missing.

4.2.3 Calculations

Flow of nutrients, 16S rRNA gene copies, and DNA
The flow of nutrients (DM, starch, nitrogen, non-starch DM), 16S rRNA gene copies and DNA were calculated by using the following equation [21]:

$$[1] \ Flow = Ti_{intake} \times \frac{Concentration_{digesta}}{Ti_{digesta}}$$

where the flow is expressed as g, copies, or mg/d, Concentration$_{digesta}$ is the concentration of nutrients (g/kg DM), 16S rRNA gene copies (copies/kg DM), or DNA (mg/kg DM) in digesta, Ti$_{digesta}$ is the titanium concentration in digesta (g/kg DM), and Ti$_{intake}$ is marker intake (g/d), which was calculated as daily targeted feed intake based on realized feed intake during frequent feeding period multiplied with the titanium concentration in feed.

Ileal and rectal starch disappearance were calculated by using the following equation [21]:

$$[2] \ Starch \ disappearance = \left(1 - \left(\frac{Starch_{digesta}}{Ti_{digesta}} \Big/ \frac{Starch_{feed}}{Ti_{feed}}\right)\right) \times 100$$

where starch disappearance is expressed as % of starch intake, Starch$_{digesta}$ is the nutrient concentration in digesta (g/kg DM), Ti$_{digesta}$ is the titanium concentration in digesta (g/kg DM), Starch$_{feed}$ is the nutrient concentration in the feed (g/kg DM), and Ti$_{feed}$ is the titanium concentration in the feed (g/kg DM). Colonic starch disappearance was calculated as the difference between total tract and ileal starch disappearance.

Starch fermentation
Starch fermentation was calculated following the method of Gerrits, *et al.* [13]. Briefly: diets were designed to have a contrast in natural ^{13}C enrichment (AP, %) between the maize starch (Waxy maize starch: 1.0937%; High amylose maize starch: 1.0937%) and the non-starch ingredients of the diet (1.0769%). Consequently, an increase in the ^{13}C enrichment in

digesta is assumed to result from a greater amount of ^{13}C from dietary starch (degradation products) in digesta, or by starch-derived ^{13}C incorporated in microbial biomass. Total starch, C content, and ^{13}C enrichment were analyzed in digesta samples to calculate the amount of starch-derived C in non-starch digesta (C-ST in NSTd; assumed to be incorporated in biomass). Finally, the amount of starch fermented was calculated by assuming that for 1 g of microbial biomass, 5 g of starch-derived C (from fermented starch) are required [13]; based on the microbial efficiency of high-starch diets in dairy cows [22] or calculated [23] by assuming a fixed conversion of carbohydrates to microbial biomass (0.3 kJ biomass/kJ carbohydrate) [24], and energy content of carbohydrates (15.56 kJ/g) [25] and biomass (23.13 kJ/g) [26].

Microbiota data analysis

Raw DNA sequence reads were processed for quality filtering, de-multiplexing, OTU-picking, and taxonomic assignment using NG-Tax [27]. An OTU-table was generated for all samples, and no rarefaction was performed to retain low-abundant taxonomic groups [28]. Alpha diversity indices, weighted- and unweighted Unifrac distances (beta-diversity), microbial biomass composition, and relative abundance of individual taxa were calculated using the Microbiome (v1.6.0, [29]) and Phyloseq (v1.28.0, [30]) packages in R (v3.5.1).

4.2.4 Statistical analysis

For statistical analyses on growth, feed intake, and flows of nutrients, 16S rRNA gene copies, DNA and C-ST in NSTd, starch disappearance, and starch fermentation, SAS 9.4 for Windows (SAS Institute, Cary, NC) was used. The pig was considered as experimental unit. All data were analyzed using a general linear mixed model (PROC MIXED),

[3] $Y_{ijk} = \mu + D_i + B_j + e_{ijk}$,

where Y is the dependent variable, μ the overall mean, D_j the fixed effect of diet (LRS or HRS), B_j the random effect of block effect which was dissection order (1-5), and e_{ijklmn} the residual error. Homogeneity and normality of model residuals were checked visually using UNIVARIATE procedure. Data are presented as LSmeans ± pooled SEM. Differences were considered significant if P < 0.05.

Pearson correlation coefficients were estimated to determine the relation between the flows of DNA, 16S rRNA gene copies, N, non-starch DM, and between the flows of C-ST in NSTd, DNA, 16S rRNA gene copies, and N in both the ileum and rectum. The Cook's distance test was used to estimate the influence of individual pigs on the outcome of each correlation. Based on its influence on predicted correlations (Cook's Distance > 1 [31]),

rectal data on DNA and 16S rRNA gene copies of one pig (LRS) were discarded before analysis.

4.3 Results

4.3.1 General

All pigs remained healthy during the entire experiment. Body weight (BW ± SD) at the start (34.9 ± 1.36 kg) and end (47.5 ± 2.83 kg) of the experiment, and daily gain (847 ± 196 g/d) did not differ between diets (Table 4.2).

4.3.2 Intestinal nutrient flow

Ileal DM flow was 239 g/d greater in HRS-fed pigs than LRS-fed pigs ($P<0.001$). Starch flow was 183 g/d greater in HRS-fed pigs ($P<0.001$) (Table 4.2). Ileal flow of non-starch DM tended to be 56 g/d greater for HRS-fed pigs than LRS-fed pigs. Rectal DM, starch, N, and non-starch DM flow were greater for HRS-fed pigs than LRS-fed pigs, 54 g/d, 1 g/d, 4 g/d, and 53 g/d, respectively.

Table 4.2 Daily gain, dry matter intake, ileal and rectal flow of dry matter, starch, nitrogen, and non-starch dry matter in growing male pigs (48±2.8 kg) fed either a diet containing 50% waxy maize starch (low resistant starch; LRS) or 50% high amylose maize starch (high resistant starch; HRS).[1]

	LRS	HRS	SEM	P-value[2]
Daily gain, g/d	819	875	64.3	0.538
DM intake, g/d	1539	1569	15.7	0.188
Ileal nutrient flow (g/d)				
Dry matter	408	647	35.7	<0.001
Starch	14.6	198	8.9	<0.001
Nitrogen	12.7	15.7	8.7	0.102
Non-starch dry matter	393	449	31	0.070
Rectal nutrient flow (g/d)				
Dry matter	248	302	10.9	0.003
Starch	1.6	2.6	0.3	0.022
Nitrogen	8.0	12.2	0.6	<0.001
Non-starch dry matter	247	300	11	0.003

[1]Data are presented as LS means ± pooled SEM; n=10 per treatment, with individual pig as the experimental unit.
[2]Model established p-values for fixed effect of diet.

4.3.3 Starch disappearance and fermentation

Ileal starch disappearance was 24 %-units greater in LRS-fed than HRS-fed pigs ($P<0.001$), whereas total tract starch disappearance did not differ between diets, implying that 24 %-units more starch disappeared in the large intestine of HRS-fed pigs (Table 4.3). Ileal flow

Chapter 4

of C-ST in NSTd was 25.5 g/d greater for HRS-fed than for LRS-fed pigs ($P=0.001$), and rectal flow C-ST in NSTd was 12.5 g/d greater for HRS-fed pigs ($P<0.001$). Total tract starch fermentation was 17.5 %-units greater in HRS-fed pigs than LRS-fed pigs.

Table 4.3 Ileal, large intestinal, and total tract starch disappearance, and total tract starch fermentation in growing male pigs (48±2.8 kg) fed either a diet containing 50% waxy maize starch (low resistant starch; LRS) or 50% of high amylose maize starch (high resistant starch; HRS).[1]

	LRS	**HRS**	**SEM**	**P-value**[2]
Starch disappearance (% of total intake)				
Ileal	98.0	73.9	1.1	<0.001
Large intestine	1.7	25.7	1.1	<0.001
Total tract	99.7	99.8	0.03	<0.001
Flow of starch-derived carbon in non-starch digesta (g/d)[3]				
Ileal	19.9	45.4	5.2	0.001
Total tract	6.5	19.0	1.48	<0.001
Total tract starch fermentation[4]				
Starch fermented, g/d	69.9	206	15.6	<0.001
Starch fermented, % of intake	9.4	26.9	1.96	<0.001

[1]Data are presented as LS means ± pooled SEM; n=10 per treatment, with individual pig as the experimental unit. [2]Model established p-values for fixed effect of diet. [3]Flow of starch-derived carbon in non-starch digesta was calculated from the contrast in natural ^{13}C enrichment between starch and non-starch dietary components [13]. [4]Starch fermentation was calculated by assuming 5 g starch fermented/g C from starch fermentation in feces [13].

4.3.4 Digesta concentration of DNA and 16S rRNA gene copies

The concentration of 16S rRNA gene copies in ileal digesta ($1.11 \cdot 10^{10} \cdot g^{-1}$ DM digesta) was smaller than in rectal digesta ($4.32 \cdot 10^{10} \cdot g^{-1}$ DM digesta) ($P<0.001$; Figure 4.1A). The DNA concentration in ileal digesta was smaller (0.08 mg/g DM digesta) than in rectal digesta (1.01 mg/g DM digesta) ($P<0.001$; Figure 4.1B). Both 16S rRNA gene copies and DNA concentrations did not differ between diets within intestinal compartments.

4.3.5 Microbial biomass composition of ileal and rectal digesta

Microbial biomass composition in rectal digesta, but not in ileal digesta, slightly differed between diets, as shown as a shift in weighted unifrac distance (Figure 4.2A) and by a greater phylogenetic diversity in LRS-fed pigs (24.8 ± 2.8) than HRS-fed pigs (19.2 ± 2.8) ($P=0.001$; Figure 4.2B). More specifically, there were notable shifts of increased Prevotellaceae ($P=0.007$) and Coriobacteriaceae ($P=0.037$) in HRS-fed pigs, and increased Streptococcaceae ($P=0.008$) and Clostridiaceae ($P=0.005$) in LRS-fed pigs (Figure 4.2C). Variation in microbial biomass composition of ileal digesta was small compared to rectal

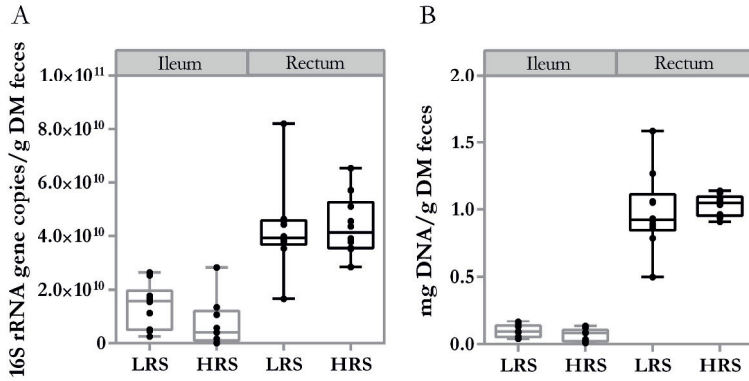digesta, with a high abundance of Lactobacillaceae and Streptococcaceae in ileal digesta (Figure 4.2C).

Figure 4.1. Concentrations of 16S rRNA gene copy numbers (A) and DNA (B) in ileal and digesta samples (per g DM feces) of growing male pigs (48±2.8 kg) fed either a diet containing 50% waxy maize starch (low resistant starch; LRS) or 50% high amylose maize starch (high resistant starch; HRS); n=9 per treatment for HRS, n=10 per treatment for LRS.
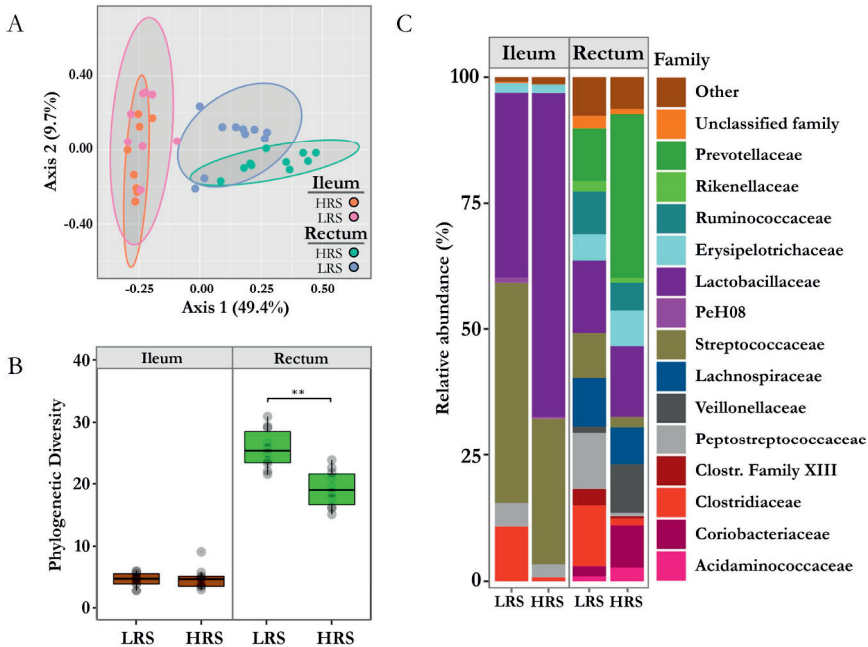

Figure 4.2 Weighted unifrac distance (A), phylogenetic diversity (B), and relative abundance of microbiota (C) in ileal and rectal digesta of growing male pigs (48±2.8 kg) fed either a diet containing 50% waxy maize starch (low resistant starch; LRS) or 50% high amylose maize starch (high resistant starch; HRS); n=9 per treatment for HRS, n=10 per treatment for LRS.

4.4 Discussion

In this study, we used the method described by Gerrits, *et al.* [13] to quantify ileal and total tract starch fermentation, assuming that all starch-derived C present in the non-starch digesta (C-ST NSTd) is originating from fermented starch. When estimated in this way, total tract starch fermentation was close to colonic starch disappearance for both HRS-fed pigs (27% compared to 26% of starch intake) and LRS-fed pigs (9% compared to 2% of starch intake), implying that the method seems valid to quantify starch fermentation from rectal digesta samples. These findings are in line with earlier studies, where 12 to 44 % of the consumed starch was fermented in the large intestine of ileal cannulated pigs fed high amylose maize starch [6, 32-34]. A minor fraction of starch was excreted with feces indicating that microbes fermented virtually all starch flowing into the large intestine. Ileal starch fermentation (HRS: 64% of starch intake; LRS: 29% of starch intake) estimated using the ileal flow of C-ST in NSTd exceeded its total tract value, indicating that ileal starch fermentation was overestimated with method used in this study. Likely, part of the C from starch measured in the ileal non-starch digesta originated from digesta material other than microbial biomass. To further investigate the overestimation of ileal starch fermentation, a post-hoc analysis was executed to determine the relation between flows of C-ST in NSTd, 16S rRNA gene copies, DNA, N, and non-starch DM (Figure 4.3).

4.4.1 Quantifying microbial biomass

The concentrations of 16S rRNA gene copies and DNA in digesta were measured as proxies of total microbial biomass [35]. The increase in both ileal and rectal flow of 16S rRNA gene copies corresponded with an increase in DNA (Figure 4.3A). This is in line with data from Miner-Williams, *et al.* [36] who showed that 85% of non-dietary DNA in ileal digesta of pigs is bacterial DNA, with the residual 15% being explained by host DNA. The zero-intercept of the relation between the ileal flow of 16S rRNA gene copies and the ileal flow of DNA suggests that the contribution of dietary DNA to ileal digesta was low. To our knowledge, the origin of fecal DNA in pigs, i.e., the distribution between dietary, microbial and host DNA, has never been reported. In humans, however, less than 1% of fecal DNA was shown to be explained by host DNA [37, 38]. In addition, endogenous and dietary components flowing into the large intestine are subjected to microbial fermentation. Free DNA from fermented cells is rapidly hydrolyzed into nucleotides by bacteria, as was shown in rumen microbiota cultures [39, 40], which are subsequently absorbed and metabolized by the host [41]. Consequently, fecal concentrations of endogenous and dietary DNA are expected to be low; hence, fecal DNA most likely largely originated from bacteria. An increase in rectal DNA flow corresponded with an increase in non-starch DM (Figure 4.3C). These results suggest that fecal output of microbial biomass strongly contributed to the greater output of

DM in HRS-fed pigs than LRS-fed pigs, which in turn, agrees with the greater total tract starch fermentation observed in HRS-fed pigs. The flow of DNA was much higher at the rectum than at the ileum, whereas the flow of 16S rRNA gene copies was relatively the same indicating that the amount of DNA with respect to the number of 16S rRNA gene copies was higher at the rectum than at the ileum of pigs (Figure 4.3A). This might be related to the difference in microbial biomass composition between ileal and rectal digesta; a bacterial cell can contain multiple 16S rRNA gene copies depending on the species [43] and possibly proliferative state. Alternatively, a greater contribution of bacterial species in the large intestine not accounted for by the RT qPCR technique, e.g., methanogenic archaea [2], may have increased the amount of DNA with respect to 16S rRNA gene copies in rectal digesta.
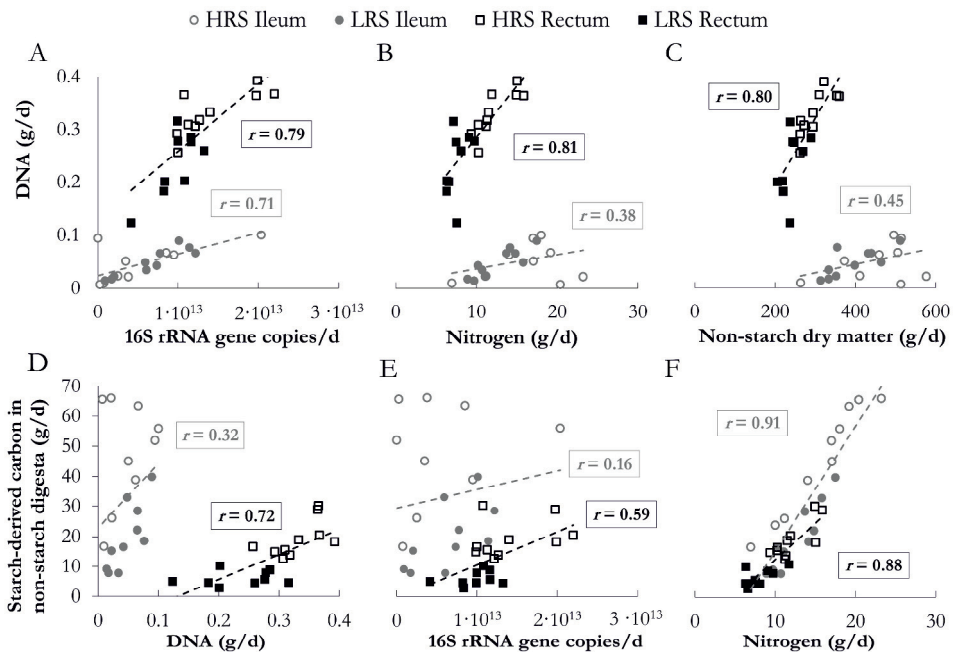
Figure 4.3 Relation between the daily ileal/rectal flow of DNA and 16rRNA gene copies (A), nitrogen (B), and non-starch dry matter (C), and between the daily ileal/rectal flow of starch-derived carbon in non-starch digesta and DNA (D), 16S rRNA gene copies (E), and nitrogen (F) in growing male pigs (48±2.8 kg) fed either a diet containing 50% waxy maize starch (low resistant starch; LRS) or 50% high amylose maize starch (high resistant starch; HRS); n=9 per treatment, for starch-derived carbon in non-starch digesta × nitrogen, n=10 per treatment.

4.4.2 Relation between ^{13}C-method and microbial biomass

For the calculation of ileal starch fermentation, we assumed that all C-ST in NSTd were incorporated into the microbial biomass. Consequently, an increase in C-ST in NSTd was expected to correspond with an increase in DNA and 16S rRNA gene copies. A relation

between these variables was observed at the rectum of pigs, but not at the ileum (Figure 4.3D and 4.3E), suggesting that at the ileum, part of the C-ST in NSTd were incorporated in digesta components other than microbial biomass. Also the flow of N was unrelated to the flow of DNA at the ileum, indicating that a greater ileal N flow did not originate from increased microbial biomass synthesis. The ileal flow of C-ST in NSTd strongly correlated with the ileal flow of N (Figure 4.3F), which may suggest that an increase in starch-derived digesta components coincided with a decrease in dietary N digestibility. Alternatively, non-dietary N-components other than microbial biomass, may have incorporated starch-derived C explaining part of the ileal flow of C-ST in NSTd. Carbons from absorbed starch-derived glucose may have been recycled by the host and incorporated into N containing components, which are subsequently secreted in the lumen, e.g., enzymes, urea, and sloughed epithelial cells [33]. The quantity of endogenous N at the ileum, however, is expected to be low as 70-80 % of the endogenous N secretion is re-absorbed up to the end of the small intestine [44, 45]. Miner-Williams*, et al.* [36] showed that mucus was the most abundant endogenous N source in ileal digesta, which contributed only 10.4% of the non-dietary nitrogen losses, another 4% was explained by urea. Starch-derived C may also have been incorporated into non-nitrogen secreted endogenous components, such as bile acids, which are absorbed by an active transport system present in the epithelium of the terminal ileum [46]. The ileal flow of C-ST in NSTd was greater in HRS-fed pigs than LRS-fed pigs, which, as explained above, was presumably unrelated to microbial biomass synthesis. Morel*, et al.* [47] showed that RS does not affect mucus secretion and ileal endogenous N losses; hence, most likely, C-ST in NSTd incorporated in endogenous losses did not contribute to the greater flow of C-ST in NSTd in HRS-fed pigs as well. Short-chain fatty acid (SCFA) concentrations, however, were shown to be increased in pigs fed high amylose maize starch [5]. An increase in unabsorbed starch-derived metabolites, like bacterial produced SCFAs, may partly explain the greater ileal flow of C-ST in NSTd in HRS-fed pigs. The strong relation between the rectal flow of N and rectal flows of DNA (Figure 4.3B) and C-ST in NSTd (Figure 4.3F) suggests that most of the fecal N output originated from bacteria. The rectal N flow was greater in HRS-fed pigs than LRS-pigs, which is possibly explained by the greater starch fermentation in HRS-fed pigs stimulating biomass synthesis. Consequently, ammonia absorption from the large intestine decreases and urea excretion from blood into the large intestine increases [9, 48], ultimately increasing fecal N excretion. The positive relation between the rectal flow of C-ST in NSTd and DNA (Figure 4.3D) and 16S rRNA gene copies (Figure 4.3E) suggests that the C-ST in NSTd were incorporated in fecal microbial biomass, which corroborates the validity of the ^{13}C-method [13] for total tract starch fermentation.

4.4.3 Ileal starch fermentation

Although we were not able to quantify ileal starch fermentation based on the ileal flow of C-ST in NSTd, it can be calculated from the difference between total tract starch fermentation (18%) and colonic starch disappearance (24%) that ileal starch fermentation was 6 %-units greater for LRS-fed pigs than HRS-fed pigs (*P*=0.046). All starch in the LRS-diet was assumed to be completely digested by host-enzymes (max *in vitro* degradation, 99.9% [34]), indicating that potentially digestible starch was fermented in the upper gastrointestinal tract of LRS-fed pigs. Our results are in line with earlier results [5, 8], that showed increased SCFA concentrations in ileal digesta of pigs fed digestible starch suggesting that part of the digestible starch was fermented. Regmi, *et al.* [5] however, reported higher SCFA concentrations in ileal digesta of pigs fed high amylose starch than in pigs fed digestible starch. SCFAs produced by bacteria early in the proximal gastrointestinal tract may already have been absorbed due to rapid absorption of SCFAs [49, 50] explaining the difference in findings between this study and Regmi, *et al.* [5]. Even in stomach contents of pigs, microbial enzyme activity was demonstrated, exceeding that of salivary amylase [51]. Because of the low competition between bacteria and host-enzymes, part of the digestible starch may already have been fermented in the stomach of LRS-fed pigs. Our results show that part of the starch is fermented in the upper gastrointestinal tract, particularly so in LRS-fed pigs, suggesting that bacteria in the upper gastrointestinal tract are opportunistic as they ferment less RS (HRS-fed pigs) than digestible starch (LRS-fed pigs).

4.4.4 Microbial biomass composition

Microbial biomass composition of ileal digesta was unaffected by diet, which is in line with earlier results reporting the ileal microbial biomass composition in pigs fed digestible starch and RS [5, 8]. Within two weeks of feeding, microbial communities and diversity in rectal digesta shifted as a response to a greater flow of undigested starch into the large intestine; bacterial alpha-diversity was lower in HRS-fed than LRS-fed pigs. Shifts in bacterial biomass composition in response to RS intake vary a lot among pig studies [5, 7, 8, 52, 53]. The major determinants of microbial biomass composition are available substrate and preferential substrate utilization of microbes [54, 55]. Consequently, the differences in microbial biomass composition among studies may be explained by the different RS sources used and the varying dietary concentrations of RS and other fermentable ingredients. In the current study, particularly the abundance of Streptococcaceae and Clostridiaceae were greater in LRS-fed than HRS-fed pigs, whereas the abundance of Prevotellaceae and Coriobacteriaceae were greater in HRS-fed pigs. Streptococcaceae has been linked to disease prevention [56] and increased growth rates in pigs [57]. The increase in saccharolytic

Chapter 4

microbes, such as Prevotellaceae [58], is probably explained by the greater availability of substrate in the large intestine of HRS-fed pigs. Increased Prevotellaceae has also been associated with increased Veillonellaceae and *Lactobacillus* [59], which were also more abundant in HRS-fed pigs than LRS-fed pigs.

4.5 Conclusions

Total tract starch fermentation was ~18 %-units greater in HRS-fed pigs than in LRS-fed pigs. As a consequence of a greater RS intake, microbial biomass composition shifted and diversity decreased in the large intestine. Microbial biomass composition in the small intestine was not affected by diet. The ^{13}C-method described by Gerrits*, et al.* [13] used to calculate starch fermentation, was reasonably close to colonic starch disappearance, which was estimated by the difference between total tract starch fermentation and ileal starch disappearance, but largely overestimated ileal starch fermentation. This was corroborated by an increase in rectal DNA flow, which was representative for microbial DNA, coinciding with an increase in the flow of starch-derived C incorporated in microbial biomass. No such relation was observed at the ileum. Our results suggest that ileal starch digestion by host-enzymes in pigs is overestimated by 1-7 % when based on ileal starch disappearance, particularly in pigs fed high digestible starch diets.

References

1. Knudsen KB, Jensen BB, Andersen J, Hansen I: Gastrointestinal implications in pigs of wheat and oat fractions: 2. Microbial activity in the gastrointestinal tract. *Br J Nutr* 1991, 65:233-248.

2. Jensen BB, Jørgensen H: Effect of dietary fiber on microbial activity and microbial gas production in various regions of the gastrointestinal tract of pigs. *Appl Environ Microbiol* 1994, 60:1897-1904.

3. Knudsen KEB, Jensen BB, Hansen I: Digestion of polysaccharides and other major components in the small and large intestine of pigs fed on diets consisting of oat fractions rich in β-D-glucan. *Br J Nutr* 1993, 70:537-556.

4. Brouns F, Kettlitz B, Arrigoni E: Resistant starch and "the butyrate revolution". *Trends Food Sci Technol* 2002, 13:251-261.

5. Regmi PR, Metzler-Zebeli BU, Gänzle MG, Van Kempen TA, Zijlstra RT: Starch with high amylose content and low *in vitro* digestibility increases intestinal nutrient flow and microbial fermentation and selectively promotes bifidobacteria in pigs. *J Nutr* 2011, 141:1273-1280.

6. Bird AR, Vuaran M, Brown I, Topping DL: Two high-amylose maize starches with different amounts of resistant starch vary in their effects on fermentation, tissue and digesta mass accretion, and bacterial populations in the large bowel of pigs. *Br J Nutr* 2007, 97:134-144.

7. Umu ÖC, Frank JA, Fangel JU, Oostindjer M, Da Silva CS, Bolhuis EJ, Bosch G, Willats WG, Pope PB, Diep DB: Resistant starch diet induces change in the swine microbiome and a predominance of beneficial bacterial populations. *Microbiome* 2015, 3:16.

8. Haenen D, Zhang J, da Silva CS, Bosch G, Van der Meer IM, Van Arkel J, Van den Borne JJ, Gutiérrez OP, Smidt H, Kemp B: A diet high in resistant starch modulates microbiota composition, SCFA concentrations, and gene expression in pig intestine. *J Nutr* 2013, 143:274-283.

9. Van der Meulen J, Bakker G, Bakker J, De Visser H, Jongbloed A, Everts H: Effect of resistant starch on net portal-drained viscera flux of glucose, volatile fatty acids, urea, and ammonia in growing pigs. *J Anim Sci* 1997, 75:2697-2704.

10. Van Kempen TA, Regmi PR, Matte JJ, Zijlstra RT: *In vitro* starch digestion kinetics, corrected for estimated gastric emptying, predict portal glucose appearance in pigs. *J Nutr* 2010, 140:1227-1233.

11. Deutz N, Ten Have G, Soeters P, Moughan P: Increased intestinal amino-acid retention from the addition of carbohydrates to a meal. *Clin Nutr* 1995, 14:354-364.

12. Vangsøe CT, Ingerslev AK, Theil PK, Hedemann MS, Lærke HN, Knudsen KEB: *In vitro* starch digestion kinetics of diets varying in resistant starch and arabinoxylan compared with *in vivo* portal appearance of glucose in pigs. *Food Research International* 2016, 88:199-206.

13. Gerrits WJ, Bosch MW, Van den Borne JJ: Quantifying resistant starch using novel, *in vivo* methodology and the energetic utilization of fermented starch in pigs. *J Nutr* 2012, 142:238-244.

14. Everts H: Energy requirement for maintenance in growing pigs. In *CVB-Documentation Report*. Wageningen, The Netherlands: Centraal Veevoeder Bureau; 2015.

15. ISO 6496: *ISO 6496: Animal feeding stuffs - Determination of moisture and other volatile matter content*. Geneva, Switzerland: International Organization for Standardization; 1999.

16. ISO 16634-1:2008: *ISO 16634-1:2008: Food products - Determination of the total nitrogen content by combustion according to the Dumas principle and calculation of the crude protein content -- Part 1: Oilseeds and animal feeding stuffs*. Geneva, Switzerland: International Organization for Standardization; 2008.

17. Van Bussel W, Kerkhof F, Van Kessel T, Lamers H, Nous D, Verdonk H, Verhoeven B, Boer N, Toonen H: Accurate determination of titanium as titanium dioxide for limited sample size

Chapter 4

digestibility studies of feed and food matrices by inductively coupled plasma optical emission spectrometry with real-time simultaneous internal standardization. *Atom Spectrosc* 2010, 31:81-88.

18. ISO 15914: *ISO 15914: Animal feeding stuffs - Determination of total starch content.* Geneva, Switzerland: International Organization for Standardization 2004.

19. Suzuki MT, Beja O, Taylor LT, DeLong EF: Phylogenetic analysis of ribosomal RNA operons from uncultivated coastal marine bacterioplankton. *Environ Microbiol* 2001, 3:323-331.

20. Claesson MJ, Wang QO, O'Sullivan O, Greene-Diniz R, Cole JR, Ross RP, O'Toole PW: Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. *Nucleic Acids Res* 2010, 38.

21. Kotb A, Luckey T: Markers in nutrition. In *Nutr Abstr Rev*1972: 813-845.

22. Lanzas C, Sniffen C, Seo Sa, Tedeschi L, Fox D: A revised CNCPS feed carbohydrate fractionation scheme for formulating rations for ruminants. *Anim Feed Sci Tech* 2007, 136:167-190.

23. Gilbert MS, Pantophlet AJ, Berends H, Pluschke AM, Van den Borne JJ, Hendriks WH, Schols HA, Gerrits WJ: Fermentation in the small intestine contributes substantially to intestinal starch disappearance in calves. *J Nutr* 2015, 145:1147-1155.

24. Livesey G: Calculating the energy values of foods: towards new empirical formulae based on diets with varied intakes of unavailable complex carbohydrates. *Eur J Clin Nutr* 1991, 45:1-12.

25. Blaxter K: *Energy metabolism in animals and man.* Cambridge (United Kingdom): Cambridge University Press; 1989.

26. Cordier J-L, Butsch BM, Birou B, von Stockar U: The relationship between elemental composition and heat of combustion of microbial biomass. *Appl Microbiol Biotechnol* 1987, 25:305-312.

27. Ramiro-Garcia J, Hermes GDA, Giatsis C, Sipkema D, Zoetendal EG, Schaap PJ, Smidt H: NG-Tax, a highly accurate and validated pipeline for analysis of 16S rRNA amplicons from complex biomes. *F1000Res* 2016, 5:1791.

28. McMurdie PJ, Holmes S: Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible. *PLoS Comput Biol* 2014, 10.

29. Tools for microbiome analysis in R. Version 1.9.1. [http://microbiome.github.com/microbiome]

30. McMurdie PJ, Holmes S: phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 2013, 8:e61217.

31. Hair A: Tatham, and Black (1998) Multivariate Data Analysis. Prentice Hall, New Jersey.

32. Martin LJ, Dumon HJ, Champ MM: Production of short-chain fatty acids from resistant starch in a pig model. *J Sci Food Agric* 1998, 77:71-80.

33. Heijnen M-LA, Beynen AC: Consumption of retrograded (RS3) but not uncooked (RS2) resistant starch shifts nitrogen excretion from urine to feces in cannulated piglets. *J Nutr* 1997, 127:1828-1832.

34. Van Erp RJJ, Kempen Tv, de Vries S, Gerrits WJJ: Pigs ferment enzymatically digestible starch when it is substituted for resistant starch *J Nutr* 2019, Accepted for publication.

35. Brukner I, Longtin Y, Oughton M, Forgetta V, Dascal A: Assay for estimating total bacterial load: relative qPCR normalisation of bacterial load with associated clinical implications. *Diagn Microbiol Infect Dis* 2015, 83:1-6.

36. Miner-Williams W, Moughan PJ, Fuller MF: Endogenous components of digesta protein from the terminal ileum of pigs fed a casein-based diet. *J Agric Food Chem* 2009, 57:2072-2078.

37. Klaassen CH, Jeunink MA, Prinsen CF, Ruers TJ, Tan AC, Strobbe LJ, Thunnissen FB: Quantification of human DNA in feces as a diagnostic test for the presence of colorectal cancer. *Clin Chem* 2003, 49:1185-1187.

38. Vincent C, Mehrotra S, Loo VG, Dewar K, Manges AR: Excretion of host DNA in feces is associated with risk of Clostridium difficile infection. *J Immunol Res* 2015, 2015.

39. Ruiz TR, Andrews S, Smith GB: Identification and characterization of nuclease activities in anaerobic environmental samples. *Can J Microbiol* 2000, 46:736-740.

40. Duggan PS, Chambers PA, Heritage J, Forbes JM: Survival of free DNA encoding antibiotic resistance from transgenic maize and the transformation activity of DNA in ovine saliva, ovine rumen fluid and silage effluent. *FEMS Microbiol Lett* 2000, 191:71-77.

41. Sanderson IR, He Y: Nucleotide uptake and metabolism by intestinal epithelial cells. *J Nutr* 1994, 124:131S-137S.

42. Murad F, Pak CY: Urinary excretion of adenosine 3′, 5′-monophosphate and guanosine 3′, 5′-monophosphate. *N Engl J Med* 1972, 286:1382-1387.

43. Větrovský T, Baldrian P: The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. *PLoS One* 2013, 8:e57923.

44. Krawielitzki K, Kreienbring F, Zebrowska T, Schadereit R, Kowalczyk J: Estimation of N absorption, secretion, and reabsorption in different intestinal sections of growing pigs using the^1^ 5N isotope dilution method. *Publication-European association for animal production* 1994, 80:79-79.

45. Souffrant W, Rerat A, Laplace J, Darcy-Vrillon B, Köhler R, Corring T, Gebhardt G, Bernard F, Jähnichen M, Schneider B: Exogenous and endogenous contributions to nitrogen fluxes in the digestive tract of pigs fed a casein diet. III. Recycling of endogenous nitrogen. *Reprod Nutr Dev* 1993, 33:373-382.

46. Hofmann AF, Eckmann L: How bile acids confer gut mucosal protection against bacteria. *Proc Natl Acad Sci USA* 2006, 103:4333-4334.

47. Morel PC, Melai J, Eady S, Coles G: Effect of non-starch polysaccharides and resistant starch on mucin secretion and endogenous amino acid losses in pigs. *Asian Australas J Anim Sci* 2005, 18:1634-1641.

48. Mosenthin R, Sauer W, Henkel H, Ahrens F, De Lange C: Tracer studies of urea kinetics in growing pigs: II. The effect of starch infusion at the distal ileum on urea recycling and bacterial nitrogen excretion. *J Anim Sci* 1992, 70:3467-3472.

49. Engelhardt W: Absorption of short-chain fatty acids from the large intestine. *Physiological and clinical aspects of short-chain fatty acids* 1995:149.

50. Kaji I, Iwanaga T, Watanabe M, Guth PH, Engel E, Kaunitz JD, Akiba Y: SCFA transport in rat duodenum. *Am J Physiol Gastrointest Liver Physiol* 2014, 308:G188-G197.

51. Martens BM, Bruininx E, Gerrits WJJ, Schols H: The importance of gastric amylases in starch digestion kinetics in pigs. In *Starch digestion kinetics in pigs: The impact of starch stucture, feed processning, and digesta passage behaviour.* Edited by Martens BM. Wageningen2019

52. Sun Y, Su Y, Zhu W: Microbiome-metabolome responses in the cecum and colon of pig to a high resistant starch diet. *Front Microbiol* 2016, 7:779.

53. Metzler-Zebeli BU, Schmitz-Esser S, Mann E, Grüll D, Molnar T, Zebeli Q: Adaptation of the cecal bacterial microbiome of growing pigs in response to resistant starch type 4. *Appl Environ Microbiol* 2015, 81:8489-8499.

Chapter 4

54.   Castillo M, Martín-Orúe S, Anguita M, Pérez J, Gasa J: Adaptation of gut microbiota to corn physical structure and different types of dietary fibre. *Livest Sci* 2007, 109:149-152.

55.   Yu M, Li Z, Chen W, Rong T, Wang G, Ma X: Microbiome-metabolomics analysis investigating the impacts of dietary starch types on the composition and metabolism of colonic microbiota in finishing pigs. *Front Microbiol* 2019, 10:1143.

56.   Ober RA, Thissen JB, Jaing CJ, Cino-Ozuna AG, Rowland RR, Niederwerder MC: Increased microbiome diversity at the time of infection is associated with improved growth rates of pigs after co-infection with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2). *Vet Microbiol* 2017, 208:203-211.

57.   Kim J, Guevarra RB, Nguyen SG, Lee J-H, Jeong DK, Unno T: Effects of the antibiotics growth promoter Tylosin on swine gut microbiota. *J Microbiol Biotechnol* 2016, 26:876-882.

58.   Tanner A, Listgarten M, Ebersole J, Strezempko M: Family V. Prevotellaceae fam. nov. *Bergey's Manual of Systematic Bacteriology: Volume 4: The Bacteroidetes, Spirochaetes, Tenericutes (Mollicutes), Acidobacteria, Fibrobacteres, Fusobacteria, Dictyoglomi, Gemmatimonadetes, Lentisphaerae, Verrucomicrobia, Chlamydiae, and Planctomycetes* 2011, 4:85.

59.   Frese SA, Parker K, Calvert CC, Mills DA: Diet shapes the gut microbiome of pigs during nursing and weaning. *Microbiome* 2015, 3:28.

Supplementary Data
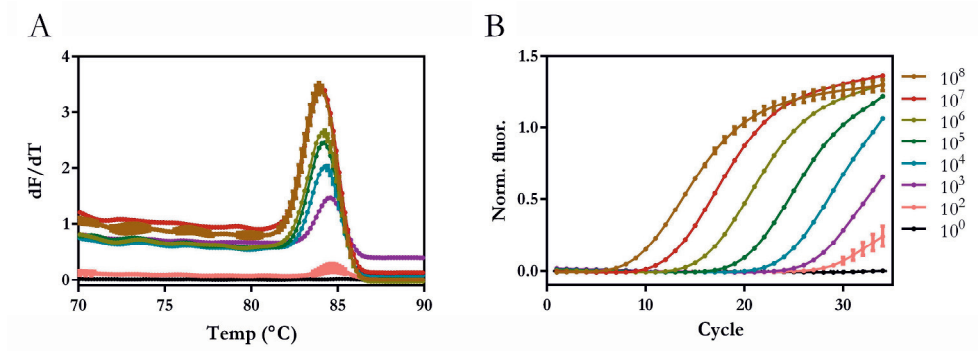
Figure S4.1 Standard curve for total 16S rRNA copy number calculations. (A) take-off curve for a mixture of Enterotoxigenic *Escherichia coli* (ETEC) and *Lactobacillus plantarum* WCSF1 (LBp), with known concentrations ranging from 10^8-10^0 CFU's. (B) melt curve of the 16S rRNA amplicon to show primer specificity.

Chapter 5

Pigs ferment enzymatically digestible starch when it is substituted for resistant starch

Rik J.J. van Erp, Sonja de Vries, Theo A.T.G. van Kempen, Walter J.J. Gerrits

Abstract

Feeding behavior is controlled by satiety mechanisms, which are affected by the extent of starch digestion, and thus resistant starch (RS) intake. Alterations in feeding behavior to changes in RS intake may depend on the adaptation of processes involved when shifting from starch digestion to fermentation or vice versa. The aim of this study was to investigate how growing pigs adapt their feeding behavior in response to increasing and decreasing dietary RS concentrations. Thirty-six groups of 6 pigs (25.4 ± 2.8 kg; Hypor Libra×Hypor Maxter; male:female, 1:1) were fed diets containing 50% high-amylose maize starch (High-RS; HRS) or waxy maize starch (Low-RS; LRS). Over 28 d, diets were exchanged following a 5-step titration (25% per step) that was executed in the upward (LH) or downward direction (HL). Twelve groups received a control diet to correct for changes over time. Individual feeding behavior and total tract starch digestion and fermentation were evaluated. The response in each parameter to increasing dietary HRS inclusion was estimated through the use of linear regression procedures, and tested for titration direction and sex effects. Complete substitution of LRS with HRS increased the proportion of starch fermented, which was greater in LH pigs than HL pigs (17.6% vs. 8.18%; P<0.001), and decreased the feed intake (106 g/d; P=0.021) and meal size (12.6 g; P<0.001) of LH pigs, but not of HL pigs. In LH pigs, the size of the starch fermentation response positively correlated with the size of the feed intake response (r = 0.90, P<0.001). The attenuated response in starch fermentation in HL pigs indicates that pigs adapt more slowly to dietary supply of digestible starch than to RS, consequently resulting in fermentation of enzymatically digestible starch. Feed intake and feeding behavior only changed in pigs poorly adapting to RS, indicating that adequacy of adaptation, rather than RS itself, drives feed intake. These findings stress the importance of diet history for nutrient digestion and feeding behavior.

5.1 Introduction

Increasing the dietary level of resistant starch (RS) at the expense of digestible starch may reduce feeding motivation in pigs [1, 2], thereby affecting their feeding behavior. The literature on the effect of RS on satiety in humans is inconclusive, as some studies report an increase in satiety following RS consumption [3-5], whereas others reported little or no effect [6, 7]. Substituting digestible starch with RS decreases postprandial glucose appearance, which reduces the release of satiety hormones (e.g.,[5, 8-10]) stimulating the ileal brake mechanism [11]. Consequently, meal size may increase, as satiation decreases. It is hypothesized, however, that the potential bulking properties of RS, similar to other fibrous feed sources [12], may increase satiation through gastric distension [13]. However, the bulking capacity of RS when compared with typical high-bulk fiber sources, such as sugar beet pulp or wheat bran, is low [12], and therefore its volumetric effect on short-term

feed intake may be limited. The presence of products from microbial fermentation in the distal gastro-intestinal tract may also slow down gastric emptying [14, 15], and activate intestinal break mechanisms [16-18], thereby decreasing digesta transit in the upper gastrointestinal tract stimulating satiation. In addition, the prolonged energy supply to the body by SCFAs resulting from microbial fermentation of RS [10] may induce long-term postprandial satiety, increasing the interval between meals.

Prolonged exposure (i.e., 12 weeks) of pigs to a high-RS diet compared with a low-RS diet increased meal size by 10% and decreased meal frequency by 2 meals/d in growing pigs, whereas daily feed intake was not affected [19]. We hypothesize, however, that these alterations in feeding behavior to changes in RS intake are dynamic, depending on the adaptation processes involved when shifting from enzymatic digestion to microbial fermentation or vice versa. To gain understanding of the mode of action of the effects of RS on feeding behavior, we investigated the effects of changes in dietary RS intake on feeding behavior of growing pigs. As these effects may depend on the direction of adaptation, we tested effects of increasing and decreasing RS concentration.

5.2 Materials & Methods

The experimental protocol was approved by the Animal Care and Use Committee of Utrecht University, Utrecht, The Netherlands; the experiment was performed at the experimental facilities of Nutreco NV, Sint Anthonis, The Netherlands.

For this study, 288 pigs (Hypor Libra × Hypor Maxter) were selected at 9 wk of age, blocked in three weight categories − light (22.2 ± 1.1 kg), medium (25.3 ± 0.8 kg) and heavy (28.7 ± 1.3 kg) − and assigned to 48 pens (6 pigs/pen) in 1 of 6 departments. Pens were 4.70 x 2.40 m with 60% slatted floors and were equipped with an electronic single-space feeding station (EFS) for fattening pigs (Schauer). Sex was equally divided over pens (1:1). After an adaptation period of 10 d during which pigs were fed a commercial diet, 36 pens (18 pens each) were assigned to 1 of 2 diets (Table 5.1), containing either 50% high amylose maize starch (high RS; HRS; Roquette) or 50% of waxy maize starch (low RS; LRS; Roquette). The rate (k; LRS: 3.14 %/min; HRS: 2.19 %/min) and extent (D; LRS: 99.9%; HRS: 65.8%) of starch digestion of each source were analyzed using an adapted *in vitro* procedure modified from Englyst*, et al.* [20], and determined by fitting the following first order kinetic model:

[1] $Starch\ degraded\ (\%\ at\ time\ t) = D \times (1 - e^{-k*t})$

Over a 28-d period, HRS and LRS were interchanged in 5 steps, either in upwards (low to high; LH) or downwards (high to low; HL) direction. The first titration step lasted for 8 d. For the successive titration steps, a length of 5 d was considered sufficient, as the increment

Chapter 5

per titration step was small. Pigs in the remaining 12 pens received a 50/50 mixture of both diets as a control treatment until the end of the experiment to control for changes in feeding behavior and digestive processes over time. Animals were exposed to 16 h of light (from 06.00h to 22.00h) and 8 h of darkness. Temperature was set at 23°C.

Table 5.1. Ingredient composition and analyzed chemical composition of experimental diets

	LRS	HRS
Ingredient (g/kg)		
Waxy maize starch[2]	500	-
High amylose maize starch[3]	-	497
Water[4]	25.5	28.5
Rape seed meal	130	130
Sunflower seed meal	130	130
Wheat gluten meal	100	100
Palm oil	33.0	33.0
Molasses, cane	25.0	25.0
Potato protein	15.0	15.0
Premix[1]	12.5	12.5
Mono-calcium phosphate	7.2	7.2
Calcium Carbonate	6.7	6.7
L-Lysine	6.0	6.0
Sodium Bicarbonate	5.5	5.5
L-Threonine	0.9	0.9
DL-Methionine	0.2	0.2
L-Tryptophan	0.2	0.2
Choline Chloride	0.1	0.1
Phytase	0.1	0.1
Titanium dioxide	2.0	2.0
Analyzed chemical composition (g/kg)		
DM	888	890
Crude fat	51.0	52.0
Crude protein	179	181
Neutral detergent fiber	102	111
Starch	444	456
Gross energy, MJ/kg	17.7	17.7

[1]DM, dry matter; HRS, high resistant starch; LRS, low resistant starch. [2]Roquette Amido di Mais Waxy N-200. [3]Roquette Amido di Mais Amylo N-400. [4]Water was included to compensate for a lower DM content of high-amylose maize starch. [5]Supplied per kg of feed: Citric acid, 111 mg; Propyl gallate, 69 mg; Butylhydroxytoluene, 151 mg; Sepiolite, 158 g; Retinyl acetate, 8,000 IU; Cholecalciferol, 1,600 IU; All-rac-α-Tocopheryl acetate, 7.5 IU; Menadione nicotinamide bisulfite, 160 mg; Thiamin mononitrate, 80 mg; Riboflavin, 400 mg; calcium-D-pantothenate 1.3 g; Choline chloride 12 g; Niacinamide 1.6 g; Pyridoxine hydrochloride, 120 mg; Folic acid, 120 mg; Cyanocobalamin, 1.6 mg; Biotin, 12 mg; Betaine hydrochloride, 7.9 mg; Iron(II)sulfate, 8 g; Calcium iodate, 80 mg; Copper(II)sulfate, 12 g; Manganese(II)oxide, 2.4 g; Zinc oxide, 8 g; Sodium selenite, 24 mg.

5.2.1 Diets and feeding

Five diets differing in LRS:HRS ratios (100:0, 75:25, 50:50, 75:25, and 0:100) were formulated to meet or exceed nutrient requirements for growing pigs [21], and pelleted. Titanium dioxide (2 g/kg) was added as indigestible marker in all diets to determine apparent total tract digestibility. During the adaptation period, pigs were fed a commercial diet (crude protein 172 g/kg, net energy 9.17 MJ/kg; ABZ). Feed and water were available *ad libitum* throughout the experiment.

5.2.2 Measurements

Pigs were weighed at the start and end of the experiment. Each pig received an electronic ear transponder corresponding to a unique identification number that was read by 2 antennas in the EFS. Data generated by the EFSs were continuously stored: the pig's identification number, the date, the time of entry and exit per visit, and amount of feed consumed per visit. On the last 2 d of each titration step, grab fecal samples were collected from the floor at 07.00h and 15.00h. All feces not visually contaminated with urine were collected to get a representative sample for all the animals in a pen. The remaining feces on the floor were removed each time after sample collection. During the other days, floors were cleaned twice a day, in the morning and evening. Samples were homogenized, pooled by pen per titration step, and stored at -20 °C. Prior to analysis, they were freeze-dried, and ground to pass a 1 mm screen. For each weight group, fecal samples of 3 randomly selected pens from each titration direction (n=9) and 2 randomly selected pens from the control group (n=6) were subsequently analyzed. Diets and feces were analyzed for contents of DM [22], nitrogen (N) and carbon (C)[23], titanium [25], and starch [24]. Only digesta samples were analyzed for starch without prior removal of soluble sugars. In starch sources, and in ball-milled non-starch diet ingredients, complete diets, and freeze-dried feces, ^{13}C-enrichment was analyzed by combustion isotope ratio MS with the use of a Delta V Advantage isotope-ratio mass spectrometer (Thermo Scientific). All analyses were carried out in duplicate.

5.2.3 Data screening and calculations

Boundaries set to clean the raw EFS data were based on visual plots of visit time × feed intake, and visit time × rate of feed intake (RFI). The registration of a visit at the EFS was considered incorrect if: visit time < 60 s and feed intake > 200 g; visit time > 60 s and rate of feed intake (RFI) > 120 g/min; visit time > 300 s and feed intake < 40 g. Applying these criteria, 557 out of 461,750 observations were discarded. Feeder visits themselves were not considered as meals, because they can occur so close together that from a digestive perspective they should be considered as a single meal. Hence, when the interval between

two successive visits did not exceed the meal criterion (320 s), visits were added together and counted as a single meal. Meal criteria were estimated individually on data obtained during the last 5 d of the pre-experimental period. Individual meal criteria were averaged to 1 meal criterion, to avoid confounding effects of individual meal criteria on meal parameter estimates. The meal criterion was estimated by fitting a model existing of 2 Gaussian and 1 Weibull distribution (equation 2) to the distribution of log-transformed intervals between 2 successive visits of each individual pig [26].

$$[2]\ y = p\left(1/\sigma_1\sqrt{2\pi}\ e^{-(x-\mu_1)^2/2\sigma_1^2}\right) + q\left(1/\sigma_1\sqrt{2\pi}\ e^{-(x-\mu_2)^2/2\sigma_2^2}\right) + (1-p-q)\left(\alpha/\beta^\alpha\right)x^{\alpha-1}e^{-(x/\beta)^\alpha}$$

Where y is the probability density of log (interval length) in seconds, p, q, and $1-p-q$ are the proportions of intervals in each distribution, χ is the log (interval length) in seconds, σ_1 and σ_2, and μ_1 and μ_2, are the respective SD and mean of the first and second distribution, and α and β are the respective scale and shape parameter of the third distribution (Figure S5.1). The first curve describes the short, within-meal intervals; the second curve is suggested to be associated with drinking behavior; the third curve describes the long intermeal intervals [26]. The intersection between the second and third curve was used to set the meal criterion [27]. Meal size and duration were calculated as the respective sum of feed intake and visit duration within 1 meal. Intermeal interval was calculated as the average time between 2 successive meals. RFI was calculated as daily feed intake divided by total time spent eating. Average daily feed intake (ADFI), RFI, time spent eating, daily number of visits, and meal frequency, size, and duration were calculated per pig, per titration step. For both titration directions, only the last 2 d of each titration step were used for calculation of the mean value per titration step. For the control group all data were used for calculations, except for the first 6 d to allow pigs to adapt to the experimental diet.

Apparent total tract digestibility (ATTD) of nutrients were calculated from the following equation [28]:

$$[3]\ Nutrient\ dissappearance\ (\%\ of\ intake) = \left(1 - \left(\frac{Nutrient_{feces}}{Ti_{feces}}\Big/\frac{Nutrient_{feed}}{Ti_{feed}}\right)\right) \times 100$$

where Nutrient$_{feces}$ is the nutrient concentration in the feces (g/kg DM), Ti$_{feces}$ is the titanium concentration in the feces (g/kg DM), Nutrient$_{feed}$ is the nutrient concentration in the feed (g/kg DM), and Ti$_{feed}$ is the titanium concentration in the feed (g/kg DM).

Total tract starch fermentation was calculated following the method of Gerrits, *et al.* [29]. Briefly: diets were designed to have a contrast in natural ^{13}C enrichment between the maize starch (Waxy maize starch: 1.0932%; High amylose maize starch: 1.0934%) and the non-starch ingredients of the diet (1.0767%). Consequently, an increase in fecal ^{13}C enrichment can result from greater fecal excretion of ^{13}C from dietary starch (degradation products), or

by starch-derived ^{13}C incorporated in microbial biomass. Total fecal starch, carbon content, and ^{13}C enrichment were analyzed to calculate the amount of starch-derived carbon incorporated in microbial biomass. Finally, the amount of starch fermented was calculated by assuming that for 1 g of microbial biomass 5 g of carbon (from fermented starch) are required [29]; this assumption is based on the microbial efficiency of high-starch diets in dairy cows [30] or calculated [31] by assuming a fixed conversion of carbohydrates to microbial biomass (0.3 kJ fecal biomass/kJ carbohydrate) [32], and energy content of carbohydrates (15.56 kJ/g) [33] and biomass (23.13 kJ/g) [34].

For each pig, the responses in feed intake, digestibility, and fermentation parameters to decreasing or increasing dietary HRS concentrations were estimated by regression procedures using SAS version 9.4 for Windows (SAS Institute) following the model:

$$[4] \; y = a + \beta x$$

Where y = mean of response variable, a = intercept (0% inclusion of HRS), β = slope of regression line, i.e., change per percentage inclusion of HRS, and x = proportion of HRS (% of total starch) or time (d). For control pigs, a similar regression was performed, taking observations at the start of the experimental period as the intercept a, and time (d) as regressor x. The calculation of responses related to increasing dietary HRS concentrations and intercepts of each pig (μ) were corrected for significant time-related changes observed in the control pigs per titration step as follows; LH: $\mu - \beta_{control}$; HL: $\mu + \beta_{control}$. The results of the control group are expressed as daily changes in all parameters, and results of LH and HL groups are expressed as the change in size of each parameter for the complete substitution of LRS with HRS (100% HRS inclusion).

5.2.4 Statistical analysis

All statistical analyses were performed with SAS version 9.4 for Windows. For all feeding behavior data, each animal was considered as an experimental unit. Response estimates for the control group (n=72), and responses of LH and HL pigs (n=108) corrected for time-related effects calculated for feeding behavior parameters were analyzed through the use of a general linear mixed model (equation 5) to check if the response was significantly different from zero. Pen was modeled as random G-side effect to account for correlation among pigs within pen. To verify linearity, individual data were checked visually, and tested for quadratic effects by adding a quadratic term to the regression model, which was significant for <15% of the population for all parameters. The homogeneity and normality of model residuals were checked visually with the UNIVARIATE procedure.

$$[5] \; Y_{ijklmn} = \mu + T_j + S_k + C_i + D_l + P_m + e_{ijklmn}$$

Where Y_{ijklm} is dependent variable (response to 100% substitution of LRS with HRS), μ is overall mean; T_j (Titration direction), S_k (Sex), C_i (Body weight class) are fixed effects; and D_l (Department) and P_m (Pen) are random effects.

Data on digestibility and fermentation parameters were measured per pen (control: $n=6$; HL: $n=9$; LH: $n=9$); therefore, the effect of sex and pen were removed from the model. Pearson correlation coefficients were estimated to investigate the relation between response parameters.

Intercepts are presented as mean ± pooled SEM. Response parameters (β) are reported as LS means ± pooled SEM, and expressed as either the increment in response parameter per day for the control pigs, or expressed as the change in the size of the parameter for the complete substitution of LRS with HRS. In this way, the responses bear the same sign, independent of titration direction, and are easier to interpret. The change in feed intake and starch fermentation of each titration direction over time (per titration step) are presented in figure S5.2 and S5.3 to exemplify the results. Differences were considered significant if $P < 0.05$.

5.3 Results

5.3.1 General

Observations of 8 pigs were discarded; 6 pigs suffered from lameness (2 control pigs, 2 LH pigs, 2 HL pigs), and 2 pigs from a rectal prolapse (1 LH pigs, 1 HL-pig). One pig died before the start of the experimental period by unknown reasons (1 control pig). Body weight did not differ among treatments, and averaged (± = standard error) 25.4 ± 0.17 kg at the start and 61.0 ± 0.36 kg at the end of the experiment. Average daily gain during the experimental period of control pigs (957 ± 11.8 g/d), HL pigs (965 ± 11.1 g/d), and LH pigs (966 ± 12.0 g/d) did not differ. Weight class did not affect any of the response parameters.

5.3.2 Digestibility parameters

Changes over time (control group)

Apparent total tract digestibility of DM and nitrogen of control pigs increased over the 28-d study period (DM: 0.08 %-unit/d, $P<0.001$; nitrogen: 0.19 %-unit/d, $P=0.001$; Table S5.1). Total tract starch digestibility increased (0.01 %-unit/d, $P=0.027$), whereas starch fermentation decreased over time (0.12 %-unit/d, $P=0.029$).
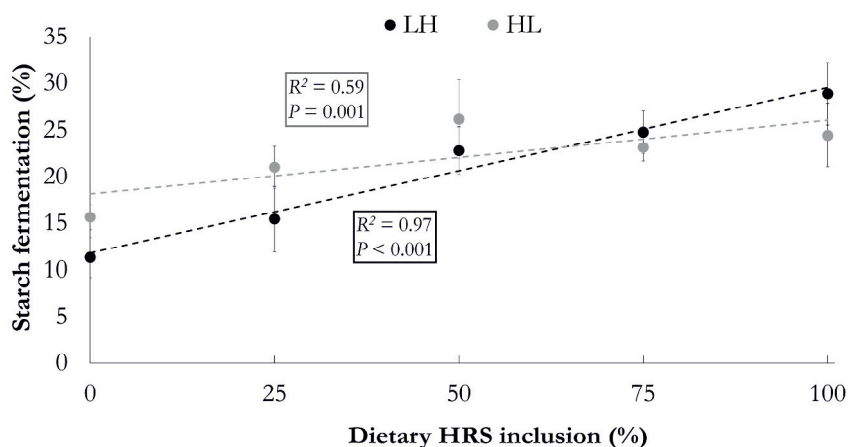
Figure 5.1 Response in dietary starch fermentation in growing pigs to incremental dietary concentrations of high amylose maize starch (HRS), when substituting waxy maize starch (LRS) with high amylose maize starch (low to high RS; LH) or vice versa (high to low RS; HL) in 5 steps from 0 to 100% over a period of 28 d. Dietary inclusion of starch sources was 50% (w/w, as fed) in all diets. Starch fermentation was calculated from the contrast in natural ^{13}C enrichment between starch and non-starch dietary components, by assuming 5 g starch fermented/g C from starch fermentation in feces [29]. Data were corrected for the time-related effect on starch fermentation measured in control groups (n=6) receiving a diet of 50% HRS and 50% LRS during the complete experiment. Data are presented as least square means ± pooled SEM, n=9 replicates (6 pigs/replicate). HL, high RS to low RS titration; HRS, high resistant starch; LH, low RS to high RS titration; LRS, low resistant starch.

Titration direction

For both LH and HL pigs, complete substitution of LRS with HRS decreased ATTD of DM, nitrogen, and starch ($P<0.010$), and increased total tract starch fermentation ($P<0.001$) (Table 5.2; Figure 5.1). Observed responses in ATTD of DM, nitrogen, and total tract starch fermentation to complete substitution of LRS with HRS were larger in LH pigs than in HL pigs ($P<0.010$), whereas the observed response in ATTD of starch digestion was smaller in LH pigs than in HL pigs ($P=0.002$).

5.3.3 Feeding behavior

Changes over time (control group)

Average daily feed intake of control animals increased by 15.1 g/d ($P<0.001$), whereas meal frequency decreased by 0.09 meal/d ($P=0.001$) (Table S5.1). This resulted in an increase of 1.72 g in meal size ($P<0.001$). Per day, meal duration increased by 0.02 min ($P<0.001$), intermeal interval by 0.49 min ($P=0.005$), and rate of feed intake by 0.27 g/min ($P<0.001$).

Chapter 5

109

Table 5.2 Intercepts and responses in nutrient disappearance and feeding behavior parameters in growing pigs (25-60 kg) to incremental dietary levels of high amylose maize (HRS), when substituting waxy maize starch (LRS) with high amylose maize starch (LH) or vice versa (HL) in 5 steps from 0 to 100% over a period of 28 d. [1]

	Intercept (0% HRS)			Response (0% HRS to 100% HRS)[2]			P-value[4]	
	LH	HL	SEM	LH[3]	HL[3]	SEM	Direction[5]	Direction × Sex
Nutrient disappearance[6] (%)								
ATTD DM	83.9	82.8	0.17	-3.82*	-2.43*	0.260	0.003	-
ATTD Nitrogen	83.3	80.2	0.30	-9.84*	-4.93*	0.468	<0.001	-
ATTD Starch	99.8	99.8	0.01	-0.14*	-0.35*	0.041	0.002	-
TT Starch fermentation[7]	11.8	18.1	0.52	17.6*	8.18*	0.013	<0.001	-
Feeding behavior[8]								
ADFI, g/d	1734	1751	35.9	-106*	-18.9	37.2	0.014	0.001
Meal frequency, n/d	18.0	19.3	0.44	0.57	-0.33	0.37	0.093	0.167
Meal size, g	103.9	91.0	3.76	-12.6*	5.82	3.14	<0.001	0.063
Meal duration, min	5.22	4.94	0.14	-0.09	0.03	0.12	0.472	0.160
Intermeal interval, min	75.3	67.1	2.11	-3.91*	3.84*	1.82	0.005	0.484
Feeding time, min/d	70.2	71.5	1.64	-0.18	-0.31	1.22	0.935	0.467
Visit frequency, n/d	43.9	44.7	1.95	0.27	2.91	1.55	0.231	0.807
Rate of feed intake, g/min	25.1	25.3	0.55	-1.19*	-0.53	0.39	0.245	0.004

[1]Data are presented as least square means ± pooled SEM. Dietary inclusion of starch sources was 50% (w/w as fed) in all diets. ATTD, apparent total tract digestibility; ADFI, average daily feed intake; HL, high RS to low RS titration; HRS, high resistant starch; LH, low RS to high RS titration; LRS, low resistant starch; TT, total tract. [2]For both titration directions, response sizes were calculated using the model $y = a + \beta x$, where y = mean of response variable, a = intercept (0% inclusion of HRS), β = response per percentage inclusion of HRS (x). Response sizes are presented as the change in each response variable corresponding to the full substitution of LRS with HRS (0% HRS to 100% HRS). Responses are corrected for the time-related effect on parameter measured in control groups (Nutrient disappearance: n=6; Feeding behavior: n=69) receiving a diet of 50% HRS and 50% LRS for the complete duration of the experiment. [3]Asterisk indicates $P<0.05$ (≠0). [4]No significant effect of body weight-class was observed. [5]Model established p-values for fixed effects of titration direction (LH or HL). [6]n=9 replicates (6 pigs/replicate). [7]Calculated from the contrast in natural ^{13}C-enrichment between starch and non-starch dietary components, by assuming 5 g starch fermented/g C from starch fermentation in feces [29]. [8]n=105 pigs.
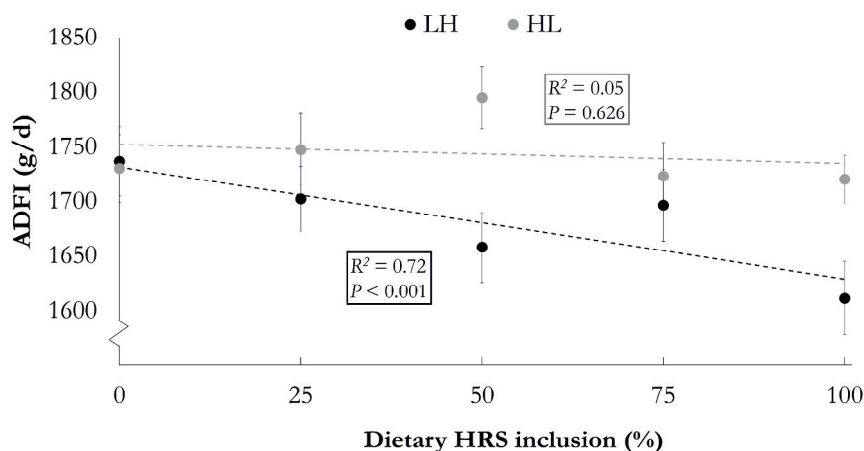
Figure 5.2 Response in ADFI of growing pigs to incremental dietary concentrations of high amylose maize starch (HRS), when substituting waxy maize starch (LRS) with high amylose maize starch (low to high RS; LH) or vice versa (high to low RS; HL) in 5 steps from 0 to 100% over a period of 28 d. Dietary inclusion of starch sources was 50% (w/w as fed) in all diets. Data were corrected for the time-related effect on parameters measured in a control groups (n=69 pigs) receiving a diet of 50% HRS and 50% LRS during the complete experiment. Data are presented as least square means ± pooled SEM, n=105 pigs. ADFI, average daily feed intake; HL, high RS to low RS titration; HRS, high resistant starch; LH, low RS to high RS titration; LRS, low resistant starch.

Titration direction

For LH pigs, the complete substitution of LRS with HRS decreased ADFI, RFI, meal size, and intermeal interval ($P<0.050$; Table 5.2; Figure 5.2). The decrease in both ADFI and RFI was greater in LH females than LH males ($P<0.010$). The decrease in meal size tended to be greater in LH females than LH males ($P=0.063$). In HL pigs, intermeal interval ($P=0.043$) increased, and daily number of visits and meal size tended to increase ($P<0.100$). Observed responses for ADFI, meal size, and intermeal interval in HL pigs were different from those in LH pigs ($P<0.050$).

5.3.4 Correlation between response parameters

In HL pigs, the response size for starch fermentation negatively correlated with the response size for ATTD of DM ($r = -0.70$, $P=0.035$) and nitrogen ($r = -0.81$, $P=0.008$). No such correlation was observed in LH pigs. In LH pigs, the response size for starch fermentation positively correlated with the response size for ADFI, whereas no correlation between these variables was observed in HL pigs (Figure 5.3).

Chapter 5
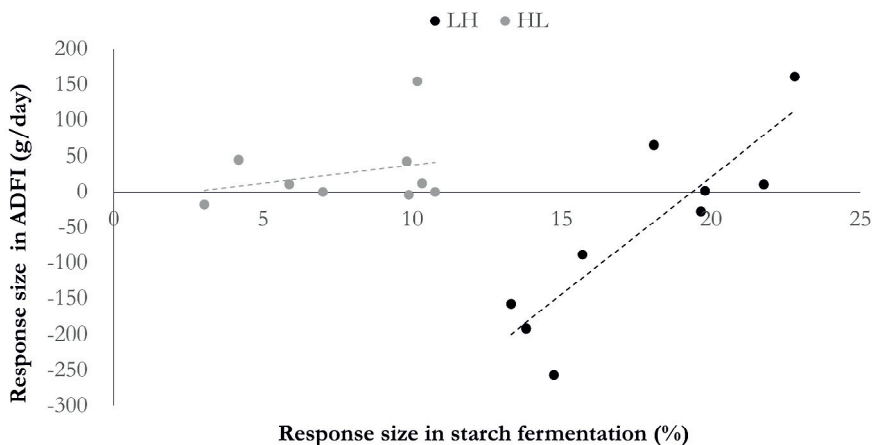
Figure 5.3. The relation between the responses in starch fermentation and ADFI to increasing dietary concentrations of high amylose maize starch (HRS) of growing pigs, which were estimated by substituting waxy maize starch (LRS) with high amylose maize starch (low to high RS; LH) or vice versa (high to low RS; HL) in 5 steps from 0 to 100% over a period of 28 d. Pearson correlation coefficients (*r*) were estimated. Dietary inclusion of starch sources was 50% (w/w as fed) in all diets fed during each step. Starch fermentation was calculated from the contrast in natural ^{13}C enrichment between starch and non-starch dietary components, by assuming 5 g starch fermented/g C from starch fermentation in feces [29]; *n*=9 replicates (6 pigs/replicate). ADFI, average daily feed intake; HL, high RS to low RS titration; HRS, high resistant starch; LH, low RS to high RS titration; LRS, low resistant starch.

5.4 Discussion

In this study, we investigated the effects of short-term changes in dietary RS on feeding behavior of pigs, by gradually substituting LRS with HRS in a 5-step titration. Titration was executed in upward and downward direction, to study anticipated differences in the adaptation process that are required to shift from enzymatic digestion to microbial fermentation and vice versa.

5.4.1 Digestive processes and feeding behavior over time

The response of the control group accounted for the anticipated increase in digestive and feeding capacity over time. The high ATTD of starch (~100%) demonstrates the large capacity of starch fermentation of the colon, resulting in very low quantities of starch excreted in the feces. Consequently, the decrease in starch fermentation over time demonstrates the adaptation of enzymatic starch digestion, leaving less starch to be fermented. This indicates that starch digestion capacity still increases in 10-wk-old pigs, which is in line with the increase in carbohydrase activity that can be observed in pigs ≤ 200 d of age [35]. Like starch, the ATTD of nitrogen increased over time, which may indicate that the enzymatic digestion capacity of nitrogen too is still increasing after 10 wk of age.

Alternatively, the increase in ATTD of nitrogen may result from the decrease in starch fermentation. Microbial fermentation increases fecal nitrogen excretion by stimulating microbial biomass formation [36], and ultimately increasing the influx of urea into the large intestine [37, 38]. The increase in feed intake, meal size, meal duration, and RFI with time, and the decrease in meal frequency with time, are in line with earlier findings [39, 40], and are presumably explained by the greater body weight, and thus, feeding capacity, as the animals grew.

5.4.2 Adaptation of digestive processes to RS intake

RS is used as a substrate for microbial fermentation [29, 36, 41] increasing starch fermentation when LRS was substituted with HRS. Increased microbial biomass [36] and urea influx [37, 38], resulting from fermentation of RS, consequently decreased the ATTD of DM and nitrogen. The responses for these parameters, however, were smaller in HL pigs than LH pigs indicating that the adaptation of the processes required to switch from starch fermentation to enzymatic starch digestion takes more time than vice versa. As a result, starch fermentation in HL pigs at the final titration step with 0% HRS was ~40% greater than initial starch fermentation at 0% HRS in LH pigs. Starch disappearance was, however, similar for both titration directions, which suggests that potentially digestible starch was fermented by HL pigs. It is possible that the increased microbial activity stimulated by high RS diets fed during the first titration steps resulted in excessive fermentation of starch during subsequent steps when low RS diets were fed. To our knowledge, this phenomenon has not yet been addressed in literature; studies into adaptation processes in pigs after dietary changes in fermentable substrate only considered increasing dietary concentrations of such substrates but not decreasing concentrations (e.g.,[42-44]). In contrast, starch fermentation in HL pigs that received a diet with 100% HRS at the initial titration step was ~15% smaller than starch fermentation in LH pigs at the final titration step (Figure 5.1). This may indicate that an 8-d adaptation before the start of the titration was insufficient for HL pigs to fully adapt to the 100% HRS diet. The time required for full adaptation to RS is difficult to establish, and the length of the period in which pigs are allowed to adapt to diets containing RS varies among studies (from 5 to 21 d) [10, 29, 36, 45]. Our results, however, show that the time required to adapt to a diet that contains RS may vary, as the rate of adaptation depends on the RS concentration in the previous diet. RS-stimulated microbiota in the small intestine still present after RS reduction probably compete with host enzymes for potentially digestible starch when switching from HRS to LRS.

5.4.3 RS intake and feeding behavior

Changes in most feed intake parameters with increasing dietary concentrations of HRS were minor (e.g., RFI and intermeal interval); however, ADFI decreased (6%) for LH pigs,

primarily in females, which coincided with a decrease in meal size (12%). In contrast, the ADFI of HL pigs did not change, consistent with a smaller increase in starch fermentation than in LH pigs. In studies by Da Silva, *et al.* [19] and Doti, *et al.* [46] daily feed intake was unaffected by dietary RS concentration (18-36 %) in growing pigs (30-110 kg), whereas meal size increased and meal frequency reduced [19]. In contrast, ingestion of RS reduced short-term food intake by 6.5% in humans [47], which was assessed by providing a test meal *ad libitum* in the evening after a fixed pre-load of RS during breakfast and lunch [48]. The difference in findings among studies may be related to the duration of RS intake, and thus to variation in the degree of adaptation to a greater dietary RS concentration. Also, the variation in extent and site of starch digestion of the various sources used may have differently affected feeding behavior [49]. The greater decrease in ADFI and meal size in female LH pigs than in male LH pigs indicates that female pigs are less capable of maintaining their feed intake when the dietary RS concentration increases, however, no evidence in pigs is available that supports this observation. In humans, however, short-chain fructo-oligosaccharides that are fermented in the large intestine reduced food intake in women, whereas in men, daily food intake was increased [50]. This may be related to a slower gastric emptying and small intestinal transit of digesta in woman than in man [51-53], enhancing the short-term satiating effect of RS, particularly in woman.

5.4.4 Relation between de degree of starch fermentation and feeding behavior

In LH pigs, the response size of ADFI was positively correlated with the response size for starch fermentation. This suggests that LH pigs that could not immediately increase starch fermentation following an increase in RS intake reduced their feed intake, whereas LH pigs that adapted more adequately were able to maintain or even increase their feed intake (Figure 5.3). These results indicate that the adequacy of adaptation, rather than the increase in dietary RS concentration itself explain the decrease in feed intake and meal size in LH pigs. The response size in total tract starch digestion in LH pigs, however, did not correlate with the response size in starch fermentation. This suggests that potentially digestible starch is fermented by LH pigs that quickly adapt to the increase in dietary RS, either because of an increased flow of digestible starch from the small intestine into the large intestine or by increased microbial activity in the small intestine.

5.5 Conclusions

Increasing dietary RS reduced enzymatic starch digestion and increased starch fermentation by 18 %-units, when LRS was completely substituted with HRS. A reverse substitution of LRS with HRS, however, attenuated these responses, indicating that pigs adapt slower to

dietary supply of digestible starch than to supply of fermentable starch. Consequently, part of the starch that appeared to be enzymatically digestible when pigs received incremental levels of RS was fermented when pigs were switched from HRS to LRS diets. These findings suggest that small intestinal fermentation may be more important than currently assumed and that the rate of adaptation to dietary changes in RS depends on diet history. In addition, feed intake and feeding behavior only changed in pigs poorly adapting to RS, indicating that adequacy of adaptation to RS, rather than RS itself, is an important driver for feed intake, and that feed intake responses to dietary RS supplementation seem rather transient. Our findings stress the importance of diet history for nutrient digestion and fermentation, and feeding behavior, and thus may have serious implications for nutrition research and advice.

Acknowledgements

Chapter 5

References

1. Bolhuis JE, Van den Brand H, Bartels AC, Oostindjer M, Van den Borne JJ, Kemp B, Gerrits WJ: Effects of fermentable starch on behaviour of growing pigs in barren or enriched housing. *Appl Anim Behav Sci* 2010, 123:77-86.

2. da Silva CS, Van den Borne JJ, Gerrits WJ, Kemp B, Bolhuis JE: Effects of dietary fibers with different physicochemical properties on feeding motivation in adult female pigs. *Physiol Behav* 2012, 107:218-230.

3. Skrabanja V, Liljeberg Elmståhl HG, Kreft I, Björck IM: Nutritional properties of starch in buckwheat products: studies *in vitro* and *in vivo*. *J Agric Food Chem* 2001, 49:490-496.

4. Willis HJ, Eldridge AL, Beiseigel J, Thomas W, Slavin JL: Greater satiety response with resistant starch and corn bran in human subjects. *Nutr Res* 2009, 29:100-105.

5. Raben A, Tagliabue A, Christensen NJ, Madsen J, Holst JJ, Astrup A: Resistant starch: the effect on postprandial glycemia, hormonal response, and satiety. *Am J Clin Nutr* 1994, 60:544-551.

6. Klosterbuer AS, Thomas W, Slavin JL: Resistant starch and pullulan reduce postprandial glucose, insulin, and GLP-1, but have no effect on satiety in healthy humans. *J Agric Food Chem* 2012, 60:11928-11934.

7. De Roos N, Heijnen M, De Graaf C, Woestenenk G, Hobbel E: Resistant starch has little effect on appetite, food intake and insulin secretion of healthy young men. *Eur J Clin Nutr* 1995, 49:532-541.

8. Van Kempen TA, Regmi PR, Matte JJ, Zijlstra RT: *In vitro* starch digestion kinetics, corrected for estimated gastric emptying, predict portal glucose appearance in pigs. *J Nutr* 2010, 140:1227-1233.

9. Noah L, Lecannu G, David A, Kozlowski F, Champ M: Digestion of starch and glycaemic response to mixed meals in pigs. *Reprod Nutr Dev* 1999, 39:245-254.

10. Regmi PR, Metzler-Zebeli BU, Gänzle MG, Van Kempen TA, Zijlstra RT: Starch with high amylose content and low *in vitro* digestibility increases intestinal nutrient flow and microbial fermentation and selectively promotes bifidobacteria in pigs. *J Nutr* 2011, 141:1273-1280.

11. Schirra J, Göke B: The physiological role of GLP-1 in human: incretin, ileal brake or more? *Regul Pept* 2005, 128:109-115.

12. De Leeuw J, Bolhuis J, Bosch G, Gerrits W: Effects of dietary fibre on behaviour and satiety in pigs: Symposium on 'Behavioural nutrition and energy balance in the young'. *Proc Nutr Soc* 2008, 67:334-342.

13. Powley TL, Phillips RJ: Gastric satiation is volumetric, intestinal satiation is nutritive. *Physiol Behav* 2004, 82:69-74.

14. Cuche G, Cuber J, Malbert C-H: Ileal short-chain fatty acids inhibit gastric motility by a humoral pathway. *Am J Physiol Gastrointest Liver Physiol* 2000, 279:G925-G930.

15. Ropert A, Cherbut C, Roze C, Le Quellec A, Holst J, Fu-Cheng X, Des Varannes SB, Galmiche J: Colonic fermentation and proximal gastric tone in humans. *Gastroenterology* 1996, 111:289-296.

16. Burcelin R: When gut fermentation controls satiety: A PYY story. *Mol Metab* 2017, 6:10-11.

17. Zhou J, Martin RJ, Tulley RT, Raggio AM, McCutcheon KL, Shen L, Danna SC, Tripathy S, Hegsted M, Keenan MJ: Dietary resistant starch upregulates total GLP-1 and PYY in a sustained day-long manner through fermentation in rodents. *Am J Physiol Endocrinol Metab* 2008, 295:E1160-E1166.

18. Pimentel M, Lin HC, Enayati P, Van den Burg B, Lee H-R, Chen JH, Park S, Kong Y, Conklin J: Methane, a gas produced by enteric bacteria, slows intestinal transit and augments small intestinal contractile activity. *Am J Physiol Gastrointest Liver Physiol* 2006, 290:G1089-G1095.

19. Da Silva CS, Bosch G, Bolhuis J, Stappers L, Van Hees H, Gerrits W, Kemp B: Effects of alginate and resistant starch on feeding patterns, behaviour and performance in *ad libitum*-fed growing pigs. *Animal* 2014, 8:1917-1927.

20. Englyst HN, Kingman S, Cummings J: Classification and measurement of nutritionally important starch fractions. *Eur J Clin Nutr* 1992, 46:S33-50.

21. Centraal Veevoeder Bureau: *CVB table pigs*. The Hague2012.

22. ISO 6496: *ISO 6496: Animal feeding stuffs - Determination of moisture and other volatile matter content.* Geneva, Switzerland: International Organization for Standardization; 1999.

23. ISO 16634-1:2008: *ISO 16634-1:2008: Food products - Determination of the total nitrogen content by combustion according to the Dumas principle and calculation of the crude protein content -- Part 1: Oilseeds and animal feeding stuffs.* Geneva, Switzerland: International Organization for Standardization; 2008.

24. ISO 15914: *ISO 15914: Animal feeding stuffs - Determination of total starch content.* Geneva, Switzerland: International Organization for Standardization 2004.

25. Van Bussel W, Kerkhof F, Van Kessel T, Lamers H, Nous D, Verdonk H, Verhoeven B, Boer N, Toonen H: Accurate determination of titanium as titanium dioxide for limited sample size digestibility studies of feed and food matrices by inductively coupled plasma optical emission spectrometry with real-time simultaneous internal standardization. *Atom Spectrosc* 2010, 31:81-88.

26. Yeates MP, Tolkamp BJ, Allcroft DJ, Kyriazakis I: The use of mixed distribution models to determine bout criteria for analysis of animal behaviour. *J Theor Biol* 2001, 213:413-425.

27. Tolkamp BJ, Kyriazakis I: To split behaviour into bouts, log-transform the intervals. *Anim Behav* 1999, 57:807-817.

28. Kotb A, Luckey T: Markers in nutrition. In *Nutr Abstr Rev* 1972: 813-845.

29. Gerrits WJ, Bosch MW, Van den Borne JJ: Quantifying resistant starch using novel, *in vivo* methodology and the energetic utilization of fermented starch in pigs. *J Nutr* 2012, 142:238-244.

30. Lanzas C, Sniffen C, Seo Sa, Tedeschi L, Fox D: A revised CNCPS feed carbohydrate fractionation scheme for formulating rations for ruminants. *Anim Feed Sci Tech* 2007, 136:167-190.

31. Gilbert MS, Pantophlet AJ, Berends H, Pluschke AM, Van den Borne JJ, Hendriks WH, Schols HA, Gerrits WJ: Fermentation in the small intestine contributes substantially to intestinal starch disappearance in calves. *J Nutr* 2015, 145:1147-1155.

32. Livesey G: Calculating the energy values of foods: towards new empirical formulae based on diets with varied intakes of unavailable complex carbohydrates. *Eur J Clin Nutr* 1991, 45:1-12.

33. Blaxter K: *Energy metabolism in animals and man.* Cambridge (United Kingdom): Cambridge University Press; 1989.

34. Cordier J-L, Butsch BM, Birou B, von Stockar U: The relationship between elemental composition and heat of combustion of microbial biomass. *Appl Microbiol Biotechnol* 1987, 25:305-312.

35. Kidder D, Manners M: The level and distribution of carbohydrases in the small intestine mucosa of pigs from 3 weeks of age to maturity. *Br J Nutr* 1980, 43:141-153.

36. Bird AR, Vuaran M, Brown I, Topping DL: Two high-amylose maize starches with different amounts of resistant starch vary in their effects on fermentation, tissue and digesta mass accretion, and bacterial populations in the large bowel of pigs. *Br J Nutr* 2007, 97:134-144.

Chapter 5

37.   Heijnen M-LA, Beynen AC: Consumption of retrograded (RS3) but not uncooked (RS2) resistant starch shifts nitrogen excretion from urine to feces in cannulated piglets. *J Nutr* 1997, 127:1828-1832.

38.   Younes H, Demigné C, Behr S, Rémésy C: Resistant starch exerts a lowering effect on plasma urea by enhancing urea N transfer into the large intestine. *Nutr Res* 1995, 15:1199-1210.

39.   Nienaber J, McDonald T, Hahn G, Chen Y: Eating dynamics of growing-finishing swine. *Transactions of the ASAE* 1990, 33:1211-1218.

40.   Hyun Y, Ellis M, McKeith F, Wilson E: Feed intake pattern of group-housed growing-finishing pigs monitored using a computerized feed intake recording system. *J Anim Sci* 1997, 75:1443-1451.

41.   Govers MJAP, Gannon NJ, Dunshea FR, Gibson PR, Muir JG: Wheat bran affects the site of fermentation of resistant starch and luminal indexes related to colon cancer risk: a study in pigs. *Gut* 1999, 45:840-847.

42.   Jonathan M, Souza da Silva C, Bosch G, Schols H, Gruppen H: Adaptation of growing pigs to diets rich in retrograded starch and/or alginate. In *Monitoring the degradation of individual dietary fibres in pig models.* Edited by Jonathan M2013: 89

43.   Castillo M, Martín-Orúe S, Anguita M, Pérez J, Gasa J: Adaptation of gut microbiota to corn physical structure and different types of dietary fibre. *Livest Sci* 2007, 109:149-152.

44.   Van Kempen TA, Kim I, Van Heugten E: Pigs as recyclers for nutrients contained in Bermuda grass harvested from spray fields. *Bioresour Technol* 2002, 81:233-239.

45.   Haenen D, Zhang J, da Silva CS, Bosch G, Van der Meer IM, Van Arkel J, Van den Borne JJ, Gutiérrez OP, Smidt H, Kemp B: A diet high in resistant starch modulates microbiota composition, SCFA concentrations, and gene expression in pig intestine. *J Nutr* 2013, 143:274-283.

46.   Doti S, Suárez-Belloch J, Latorre M, Guada J, Fondevila M: Effect of dietary starch source on growth performances, digestibility and quality traits of growing pigs. *Livest Sci* 2014, 164:119-127.

47.   Wanders AJ, Van den Borne JJ, de Graaf C, Hulshof T, Jonathan MC, Kristensen M, Mars M, Schols HA, Feskens EJ: Effects of dietary fibre on subjective appetite, energy intake and body weight: a systematic review of randomized controlled trials. *Obes Rev* 2011, 12:724-739.

48.   Bodinham CL, Frost GS, Robertson MD: Acute ingestion of resistant starch reduces food intake in healthy adults. *Br J Nutr* 2010, 103:917-922.

49.   Woltman T, Reidelberger R: Effects of duodenal and distal ileal infusions of glucose and oleic acid on meal patterns in rats. *Am J Physiol Regul Integr Comp Physiol* 1995, 269:R7-R14.

50.   Hess JR, Birkett AM, Thomas W, Slavin JL: Effects of short-chain fructooligosaccharides on satiety responses in healthy men and women. *Appetite* 2011, 56:128-134.

51.   Mori H, Suzuki H, Matsuzaki J, Taniguchi K, Shimizu T, Yamane T, Masaoka T, Kanai T: Gender difference of gastric emptying in healthy volunteers and patients with functional dyspepsia. *Digestion* 2017, 95:72-78.

52.   Sadik R, Abrahamsson H, Stotzer P-O: Gender differences in gut transit shown with a newly developed radiological procedure. *Scand J Gastroenterol* 2003, 38:36-42.

53.   Bennink R, Peeters M, Van den Maegdenbergh V, Geypens B, Rutgeerts P, De Roo M, Mortelmans L: Evaluation of small-bowel transit for solid and liquid test meal in healthy men and women. *Eur J Nucl Med* 1999, 26:1560-1566.

Supplementary Data

Table S5.1 Responses in nutrient disappearance and feed intake behavior per day in growing pigs fed a diet containing 50% waxy maize starch and 50% high amylose maize during a period of 28 days (control group). Data is presented as least square means ± SEM[1,2]

	Intercept (day 0)			Response (Per day)[6]			*P*-value[7,8] BW-class
Nutrient disappearance (%)[3]							
ATTD DM	82.1	±	0.14	0.08*	±	0.007	0.001
ATTD Nitrogen	78.0	±	0.68	0.19*	±	0.013	0.001
ATTD Starch	99.7	±	0.01	0.01*	±	0.001	0.011
TT Starch fermentation[5]	21.7	±	0.81	-0.12*	±	0.031	0.029
Feed intake behaviour[4]							
ADFI, g/d	1700	±	28.5	15.1*	±	1.41	0.410
Meal frequency, n/d	18.0	±	0.51	-0.09*	±	0.02	0.307
Meal size, g	102	±	3.91	1.72*	±	0.18	0.078
Meal duration, min	5.37	±	0.17	0.02*	±	0.00	0.298
Intermeal interval, min	76.4	±	2.76	0.49*	±	0.13	0.161
Feeding time, min/d	72.6	±	1.24	-0.10	±	0.05	0.818
Visit frequency, n/d	44.0	±	1.89	-0.05	±	0.06	0.029
Rate of feed intake, g/min	24.3	±	0.53	0.27*	±	0.02	0.504

[1]ATTD, apparent total tract digestibility; ADFI, average daily feed intake; TT, total tract. [2]Dietary inclusion of starch sources was 50% (w/w as fed). [3]n=6 replicates (6 pigs/replicate); [4]n=69 pigs. [5]Calculated from the contrast in natural ^{13}C-enrichment between starch and non-starch dietary components, by assuming 5 g starch fermented/g C from starch fermentation in feces [29]. [6]Asterisk indicates *P*<0.05 ($\mu \neq 0$). [7]Model established p-values for fixed effects of body weight class. [8]No significant effect of sex was observed.
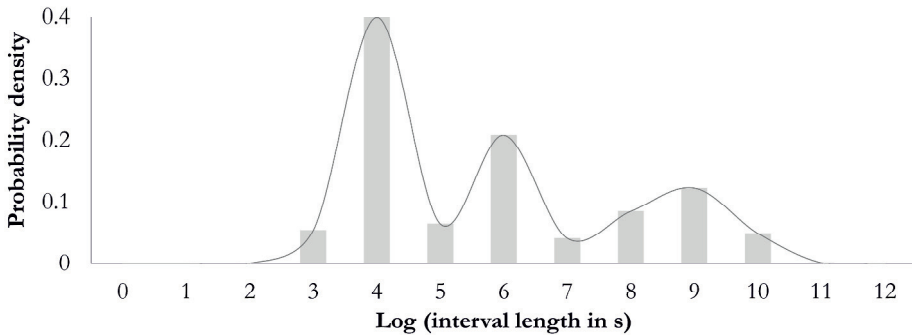
Figure S5.1 Typical probability density function of the model:

$$y = p\left(1/\sigma_1\sqrt{2\pi}\ e^{-(x-\mu_1)^2/2\sigma_1^2}\right) + q\left(1/\sigma_1\sqrt{2\pi}\ e^{-(x-\mu_2)^2/2\sigma_2^2}\right) + (1-p-q)\left(\alpha/\beta^\alpha\right)x^{\alpha-1}e^{-(x/\beta)^\alpha}$$

where y is the probability density of log (interval length) in seconds, p, q, and 1-p-q are the proportions of intervals in each distribution, χ is the log (interval length) in seconds, σ1 and σ2, and μ1 and μ2, are the respective standard deviation and mean of the first and second distribution, and α and β are the respective scale and shape parameter of the third distribution.

Chapter 5
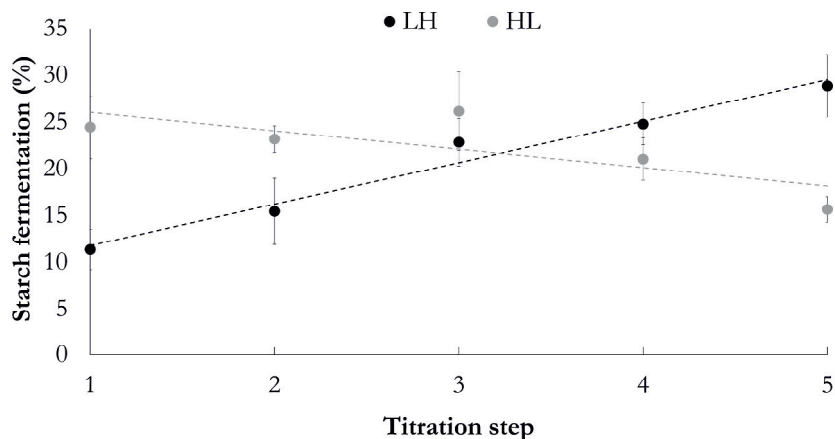
Figure S5.2 Response in dietary starch fermentation in growing pigs per titration step, when substituting waxy maize starch (LRS) with high amylose maize starch (low to high RS; LH) or vice versa (high to low RS; HL) in 5 steps from 0-100% over a period of 28 days. Dietary inclusion of starch sources was 50% (w/w, as fed) in all diets. Starch fermentation was calculated from the contrast in natural ^{13}C-enrichment between starch and non-starch dietary components, by assuming 5 g starch fermented/g C from starch fermentation in feces [29]. Data were corrected for the time-related effect on starch fermentation measured in control groups (n=6) receiving a diet of 50% waxy maize starch and 50% high amylose maize starch during the complete experiment. Data are presented as least square means ± SEM, n=9 replicates (group of 6 pigs).
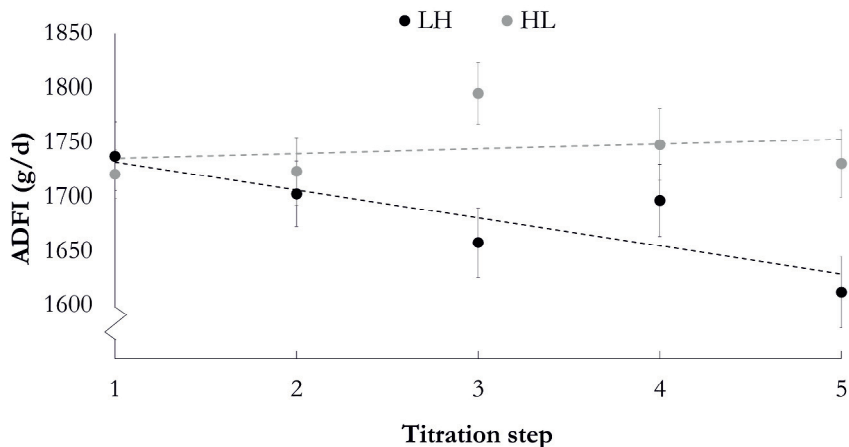
Figure S5.3 Response in average daily feed intake (ADFI) of growing pigs per titration step, when substituting waxy maize starch with high amylose maize starch (low to high RS; LH) or vice versa (high to low RS; HL) in 5 steps from 0-100% over a period of 28 days. Dietary inclusion of starch sources was 50% (w/w as fed) in all diets. Data were corrected for the time-related effect on parameters measured in a control groups (n=69 pigs) receiving a diet of 50% waxy maize starch and 50% high amylose maize starch during the complete experiment. Data are presented as least square means ± SEM, n=105 pigs.

Chapter 6

Circadian misalignment imposed by nocturnal feeding increases fat deposition in pigs

Rik J.J. van Erp, Sonja de Vries, Theo A.T.G. van Kempen, Leo A. Den Hartog, Walter J.J. Gerrits

Abstract

Misalignment of day/night and feeding rhythms have been shown to increase fat deposition and the risk for metabolic disorders in humans and rodents. In most studies, however, food intake and intake patterns are not controlled. We studied the effects of circadian misalignment on energy expenditure in pigs in a setting in which we controlled food intake as well as intake patterns. Twelve groups of five male pigs were housed in respiration chambers and fed either during the day (10.00h – 18.00h; diurnal feeding: DF) or night (22.00h – 06.00h; nocturnal feeding: NF), bihourly the same sequential meals, representing 15, 10, 25, 30 and 20% of the daily allowance. Paired feeding was applied to ensure equal gross energy intake between treatments. Apparent total tract digestibility, energy balances, and heat partitioning were measured, and analyzed using a mixed linear model. Apparent total tract energy and dry matter digestibility tended to be lower for NF-pigs than DF-pigs (P<0.10). Heat production was 3% lower for NF-pigs than DF-pigs (P<0.026) increasing fat retention by 7% in NF-pigs (P=0.050). Nocturnal-fed pigs were less active than DF-pigs during the feeding period, but more active during the fasting period. Resting metabolic rate was greater for DF-pigs than NF-pigs during the fasting period. Methane production was 30% greater in NF-pigs than in DF-pigs (P<0.001). In conclusion, circadian misalignment has little effect on nutrient digestion, but alters nutrient partitioning, ultimately increasing fat deposition. The causality of the association between circadian misalignment and methane production rates remains to be investigated.

6.1 Introduction

The timing of food intake in humans and animals is triggered by energy-deficiency signals, and simultaneously constrained by the endogenous circadian clock [1]. The endogenous circadian clock is entrained by environmental factors, so called zeitgebers, with as most important zeitgeber the light/dark cycle [2]. In humans that deviate from their normal circadian rhythm due to e.g., shift work, (social) jet lag, or night eating syndrome, the circadian eating pattern does not align with the endogenous circadian clock. This can be referred to as a form of circadian misalignment. Recurrent exposure to this phenomenon increases the risk for metabolic disorders like obesity [3-6], reduced insulin resistance [7], diabetes type 2 [8, 9], or cardiometabolic diseases [10, 11].

Like humans, rodents, which are nocturnal animals, gain more weight [12-14] and abdominal fat [12, 14] when fed during the day (non-active period) instead of the night. Increased body weights in humans and rodents that are exposed to circadian misalignment may be explained by a greater caloric intake [15] or reduced heat loss [13, 16]. Also disruptions in the adipocyte-specific circadian clock may play a role [17]. In addition, humans have a greater preference for fat-rich food at dinnertime than at breakfast time [18], which may be associated with obesity associated with night-eat syndrome [19]. However, in most of these studies that investigate the consequences of circadian misalignment, food intake and food intake patterns are not controlled. In addition, pigs are suggested to be a more suitable model for digestive strategies in humans than rodents [20], because the dietary habits of pigs more closely resemble those of humans; meal-eaters (pigs and humans) compared to nibblers (rats) [21]. Besides the similarities in anatomy, physiology, and metabolism between pigs and humans, also the microbiome of pigs is more similar to humans than that of rodents [20]. In addition, in common pig production systems, pigs, like humans, deviate from their circadian feeding pattern under certain circumstances, for example when exposed to high temperatures [22, 23] or when forced by increased feeding competition between animals housed in the same pen [24].

To disentangle the effects of dietary intake preferences and caloric intake from the effects of circadian misalignment, we investigated the effect of circadian misalignment induced by nocturnal feeding on energy metabolism in pigs using respiration chambers. Based on earlier results in humans and rodents, we hypothesize that asynchronization of the endogenous circadian clock and the timing of feed intake stimulates fat deposition in growing pigs.

6.2 Materials & Methods

The experimental protocol was approved by the Animal Care and Use Committee of Wageningen University; the experiment was performed at the experimental facilities of Wageningen University, Wageningen, the Netherlands.

6.2.1 Experimental design

Sixty male pigs (42 ±1.8 kg; TOPIGS TEMPO x TOPIGS70) were assigned to one of two treatments; receiving feed during the day (diurnal feeding; DF) or night (nocturnal feeding; NF). Pigs were group housed (n=5), which allowed for normal circadian oscillations in hormone levels to occur, like e.g., cortisol [25] that plays an important role in the regulation of circadian feeding rhythms of pigs [26]. Due to the simultaneous availability of 4 climate controlled respiration chambers, the experiment was carried out in three successive batches, which each batch consisting of a 7-day (NF-group) or 8-day (DF-group) period wherein pigs were allowed to adapt to housing conditions and experimental treatments, followed by a 7-day experimental period. Paired feeding was applied to ensure equal energy intake between treatments: DF-pigs were fed the same amount of feed as was consumed by NF-pigs the previous night. Consequently, the experimental period started one day later for DF-pigs than for NF-pigs. Each chamber [27] contained one pen of 1.75 × 2.85 m with 40% slatted floor. Temperature was maintained at 20°C and relative humidity at 65%. During the adaptation and experimental periods, pigs were exposed to 12 h of light (from 08.00h to 20.00h) and 12 h of darkness. All windows were covered to prevent exposure to light from outside. Dim light (3 lux) was placed above the feeding trough to ensure visibility of feed at night (NF-pigs).

6.2.2 Diets and feeding

Pigs of both treatments were fed the same pelleted diet (Table 6.1), representative for standard commercial practice, which was formulated to meet or exceed the nutrient requirements for growing pigs [28]. Titanium dioxide (2 g/kg dry matter) was added as indigestible marker to measure nutrient digestibility. Each group of DF-pigs was paired with a group of NF-pigs and fed the same amount of feed per kg $BW^{0.6}$ [29] as consumed by their paired group during the night before. At the start of the experiment, level of feed supplied per day was set at 1.6 times the energy requirements of pigs for maintenance (750 kJ NE·kg $BW^{-0.6}$; CVB, 2017), and, within each pair, this was increased with 75 kJ NE·kg $BW^{-0.6}$ per day when no feed residues were collected two hours after the last meal. For both treatments, feed allowance was adjusted daily, based on body weight and expected daily gain that was calculated by dividing daily feed intake by a fixed feed to gain ratio of 1:2.3 g/g. From day 1 onwards, nocturnal-fed pigs were fed bihourly between 22.00h and 06.00h resulting in five successive meals (Figure 6.1). Meals had a respective size of 15%, 10%, 25%, 30%, and 20% (wt./wt.) of the daily feed allowance, which was based on the bimodal rhythm in feeding patterns of domestic pigs that have *ad libitum* access to feed [30], which corresponds with activity patterns of wild boars in nature [31, 32]. Diurnal-fed pigs were fed

according to the same feeding schedule exactly 12 hours later than NF-pigs between 10.00h and 18.00h (Figure 6.1). Water was available *ad libitum* throughout the entire study period.

Table 6.1 Ingredient composition and analyzed chemical composition of experimental diets

	g/kg
Ingredients	
Wheat	577
Soybean meal	180
Barley	138
Wheat bran	48.5
Soybean oil	23.4
Premix[1]	12.5
Calcium Carbonate	9.46
Sodium Chloride	2.70
Mono-calcium phosphate	2.62
L-Lysine	1.17
Choline Chloride	1.09
Phytase	0.41
DL-Methionine	0.31
All-rac-α-Tocopheryl acetate	0.26
L-Valine	0.20
Sodium Bicarbonate	0.16
L-Threonine	0.10
L-Tryptophan	0.02
Titanium dioxide	2.00
Analyzed chemical composition	
Dry matter, g/kg	884
Crude protein, g/kg	173
Energy, MJ/kg	16.6

[1]Supplied per kg of feed: Citric acid, 111 mg; Propyl gallate, 69 mg; Butylhydroxytoluene, 151 mg; Sepiolite, 158 g; Retinyl acetate, 8,000 IU; Cholecalciferol, 1,600 IU; All-rac-α-Tocopheryl acetate, 7·5 IU; Menadione nicotinamide bisulfite, 160 mg; Thiamin mononitrate, 80 mg; Riboflavin, 400 mg; calcium-D-pantothenate 1·3 g; Choline chloride 12 g; Niacinamide 1·6 g; Pyridoxine hydrochloride, 120 mg; Folic acid, 120 mg; Cyanocobalamin, 1·6 mg; Biotin, 12 mg; Betaine hydrochloride, 7·9 mg; Iron(II)sulfate, 8 g; Calcium iodate, 80 mg; Copper(II)sulfate, 12 g; Manganese(II)oxide, 2·4 g; Zinc oxide, 8 g; Sodium selenite, 24 mg.

6.2.3 Measurements

Pigs were weighed before and after the experimental period. During the experimental period, energy and nitrogen balances were measured per group of pigs. Feed was sampled daily. At the end of the experimental period, each room was thoroughly cleaned and mixtures of feces, urine, and cleaning water were quantitatively collected, homogenized, sampled, and stored at -20 °C. NH_3 in excurrent air was quantitatively trapped in water condensed on the heat exchanger or in 25% sulfuric acid solution. Exchange of O_2, CO_2, and CH_4 was recorded in 9 min intervals. A CO_2 recovery test was performed as a full

Chapter 6

system check, prior to the start of the experiment [33]. In the four chambers, 100.3%, 100.2%, 100.3%, and 99.9% of the CO_2 released was recovered. Per group of pigs, physical activity was recorded continuously by a radar device. At d 4, 5, and 6 of the experimental week, fecal grab samples, not visually contaminated with urine, were collected at 08.00h and 20.00h, weighed and stored at 4 °C. At the end of the week, grab samples were homogenized, pooled, and stored at -20 °C. Samples of collected mixed feces and urine and grab feces were freeze-dried and ground to pass a 1 mm screen (Retsch ZM200, Haan, Germany). Feed samples were pooled per group of pigs and ground to pass a 1 mm screen (Retsch ZM200, Haan, Germany). Dry matter (DM) was analyzed in feed, mixed feces and urine, grab feces, freeze-dried mixed feces and urine, and freeze-dried grab feces [34]. Gross energy was analyzed in feed, freeze-dried mixed feces and urine, and freeze-dried grab feces using bomb calorimetry [35]. Nitrogen was analyzed in feed, mixed feces and urine, grab feces, water condensate, and 25% sulfuric acid solution using the Kjeldahl method [36]. Titanium was analyzed in feed and freeze-dried grab feces [37]. All analyses were performed in duplicate.
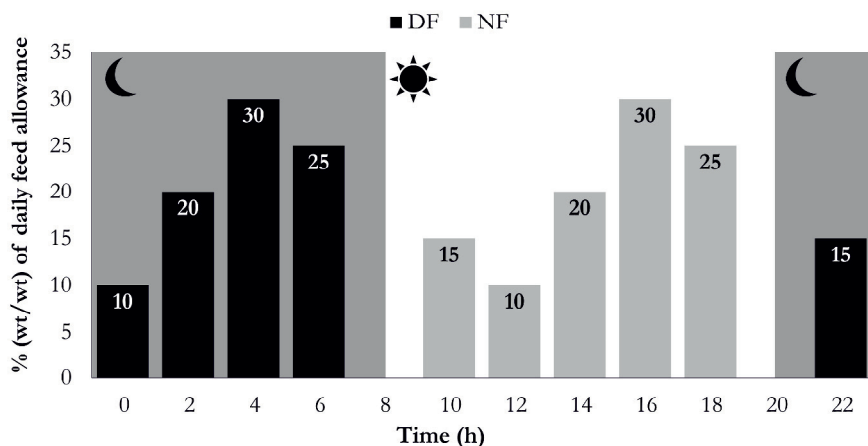
Figure 6.1 Feeding schedule of growing pigs fed five meals bihourly during the day (10.00h – 18.00h; Diurnal feeding, DF) or night (22.00h – 06.00h; Nocturnal feeding; NF). The dark area represents the period where the lights were switched off.

6.2.4 Calculations

Energy intake was calculated by multiplying daily feed intake by its gross energy (GE) content. Metabolizable energy (ME) was calculated by subtracting energy losses in mixed feces and urine, grab feces, and methane, from GE intake. Heat production was calculated with Brouwer's equation without protein coefficients [38]. Energy deposition was calculated by subtracting heat production from ME intake. Nitrogen (N) deposition was calculated as N intake - N losses via mixed feces and urine, grab feces, and NH_3 in exhaust air captured

in sulfuric acid solution and water condensate. Energy deposited as protein was calculated as N deposition × 6.25, and multiplied by 23.7 kJ/g to calculate energy deposited as protein. Energy deposited as fat was calculated as the difference between energy deposited and energy deposited as protein. Values are expressed as kJ·kg $BW^{-0.6}$·d^{-1} [29]. Net carbohydrate (CHO) and fatty acid (FA) oxidation were calculated from gas exchange measurements, without protein and methane coefficients [39]. Respiratory quotient (RQ) was calculated as CO_2 produced divided by O_2 consumed. Resting metabolic rate (RMR) and heat production related to activity were estimated from total heat production and activity data using penalized b-spline regression procedures [40]. Activity counts measured by one of the radar devices, which was used in one of the chambers of each batch (2 NF; 1 DF), were cut off to a value of 100 before activity heat was estimated. Apparent total tract digestibility of nutrients were calculated using the following equation [41]:

$$[1] \; Nutrient \; dissappearance \; (\% \; of \; intake) = \left(1 - \left(\frac{Nutrient_{feces}}{Ti_{feces}} \bigg/ \frac{Nutrient_{feed}}{Ti_{feed}}\right)\right) \times 100$$

where $Nutrient_{feces}$ is the nutrient concentration in grab feces (g/kg DM), Ti_{feces} is the titanium concentration in grab feces (g/kg DM), $Nutrient_{feed}$ is the nutrient concentration in the feed (g/kg DM), and Ti_{feed} is the titanium concentration in the feed (g/kg DM).

6.2.5 Statistics

For all statistical analyses, SAS 9.4 for Windows (SAS Institute, Cary, NC) was used. A group of five pigs housed within one chamber was considered as the experimental unit. Data from energy balance, apparent total tract digestibility, heat production, RQ, and net CHO and fat oxidation were analyzed using a general linear mixed model [2].

[2] $Y_{ijk} = \mu + T_i + B_j + P_k + e_{ijkl}$,

where Y = dependent variable; μ = overall mean; T_i = fixed effect of diurnal or nocturnal feeding; B_j = block effect batch 1, 2, or 3; P_k = random effect of pair; and e_{ijkl} = residual error. Interaction between fixed effects T_i and B_j was checked, and deleted from the model when not significant ($P<0.05$). Diurnal patterns of total heat production, activity heat, RQ, and net CHO and fat oxidation were analyzed by using the same general linear mixed model by hour. Diurnal patterns were aligned per feeding time. In this way, the responses to each meal bear the same sign, which makes it possible to compare them. Homogeneity of variances and normality of model residuals was checked visually using the UNIVARIATE procedure. Differences were considered significant if $P<0.05$. Data are reported as LS means ± SEM.

Chapter 6

6.3 Results

6.3.1 General

Throughout the study, two pigs were removed before the start of the experimental week, and one pig was removed during the balance week; all three pigs were removed because of fever (body temperature > 40 °C). Weight gain during the experimental period of NF-pigs was greater than DF-pigs (790±40.5 g/d vs. 660±40.5 g/d; P=0.007).

Table 6.2 Energy partitioning and efficiency, nitrogen efficiency, substrate oxidation, and apparent total tract digestibility of dry matter, nitrogen, and energy in pigs fed bihourly five successive meals either during the day (10.00h – 18.00h; diurnal feeding, NF) or night (22.00h – 06.00h; nocturnal feeding, NF).[1,2,3]

	DF	NF	SEM[1]	P-value[4] Diet
Energy balance (kJ · kg BW$^{-0.6}$ · d^{-1})				
Energy intake	2447	2449	63.3	0.878
Feces + urine	377	378	15.9	0.906
Methane	6.68	8.74	0.92	<0.001
ME intake	2063	2063	49.0	0.967
Heat production	1269	1230	19.0	0.026
Resting metabolic rate	1056	1032	16.1	0.216
Activity heat	213	198	16.1	0.440
Energy deposition	795	833	31.7	0.066
As protein	290	293	9.72	0.557
As fat	505	540	22.5	0.050
Energy efficiency, %	32.4	34.0	0.52	0.061
Nitrogen efficiency, %	47.8	48.3	0.55	0.550
Net substrate oxidation				
RQ	1.062	1.065	0.003	0.326
Carbohydrate, g · kg BW$^{-0.6}$ · d^{-1}	86.4	84.6	1.88	0.006
Fat, g · kg BW$^{-0.6}$ · d^{-1}	-6.31	-6.49	0.40	0.427
Apparent total tract nutrient digestibility (%)				
Dry matter	87.1	86.6	0.19	0.068
Energy	86.2	85.6	0.23	0.072
Nitrogen	84.0	83.2	0.39	0.224

[1]Values are presented as least square means and pooled standard errors of the mean (SEM), n=6, with each experimental unit being a group of 5 male pigs. [2]ME, Metabolizable energy; RQ, Respiratory quotient. [3]Subsequent meals had a size of 15%, 10%, 25%, 30%, and 20% of the daily feed allowance. [4]No significant effect of batch was observed.

6.3.2 Energy balance and nutrient digestibility

Energy intake did not differ between treatments, indicating that paired feeding was executed successfully. Apparent total tract digestibility of DM and energy tended to be greater for

DF-pigs than NF-pigs ($P<0.100$; Table 6.2). Energy losses with mixed feces and urine were similar, whereas methane production was 2.06 kJ·kg $BW^{-0.6}$ smaller for DF-pigs than NF-pigs ($P<0.001$). The difference in methane production remained constant during 24 h (Figure 6.2). Metabolizable energy intake was similar for both treatments. Heat production was 39 kJ·kg $BW^{-0.6}$ smaller for DF-pigs than NF-pigs ($P=0.026$). Energy deposition tended to be smaller for DF-pigs than NF-pigs (38 kJ·kg $BW^{-0.6}$, $P=0.066$). Energy deposited as fat was 35 kJ·kg $BW^{-0.6}$ smaller for DF-pigs than NF-pigs ($P=0.050$), whereas energy deposited as protein was similar between treatments. Energy efficiency tended to be 1.6% greater for NF-pigs than DF-pigs ($P=0.061$).
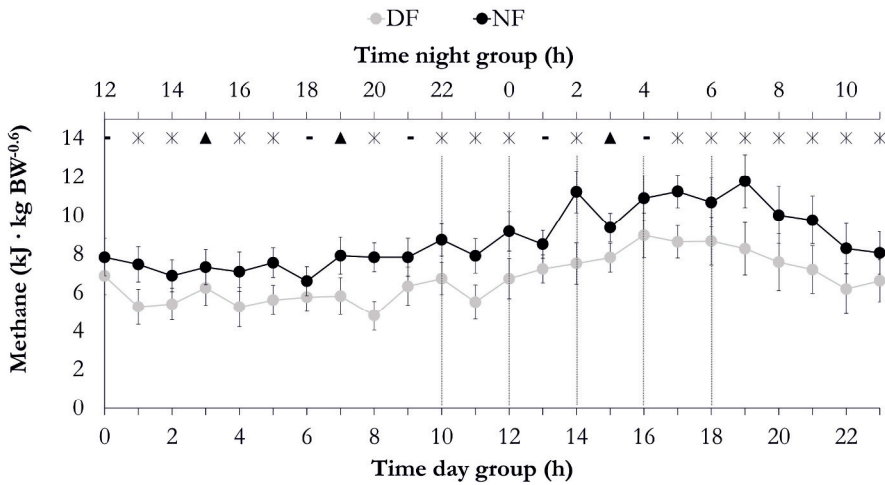
Figure 6.2 Circadian pattern of energy losses through methane production in male pigs (BW 42 ±1.8 kg) when feeding five meals bihourly during the day (10.00h – 18.00h; bottom horizontal axis) or night (22.00h – 06.00h; top horizontal axis). Dotted lines represent feeding times. Values are presented as least square means and pooled standard error of the mean, n=6 with each experimental unit being a group of 5 male pigs. ✳ represents $P < 0.05$, ▲ represents $0.05 < P < 0.1$, - represents $P > 0.1$.

Chapter 6
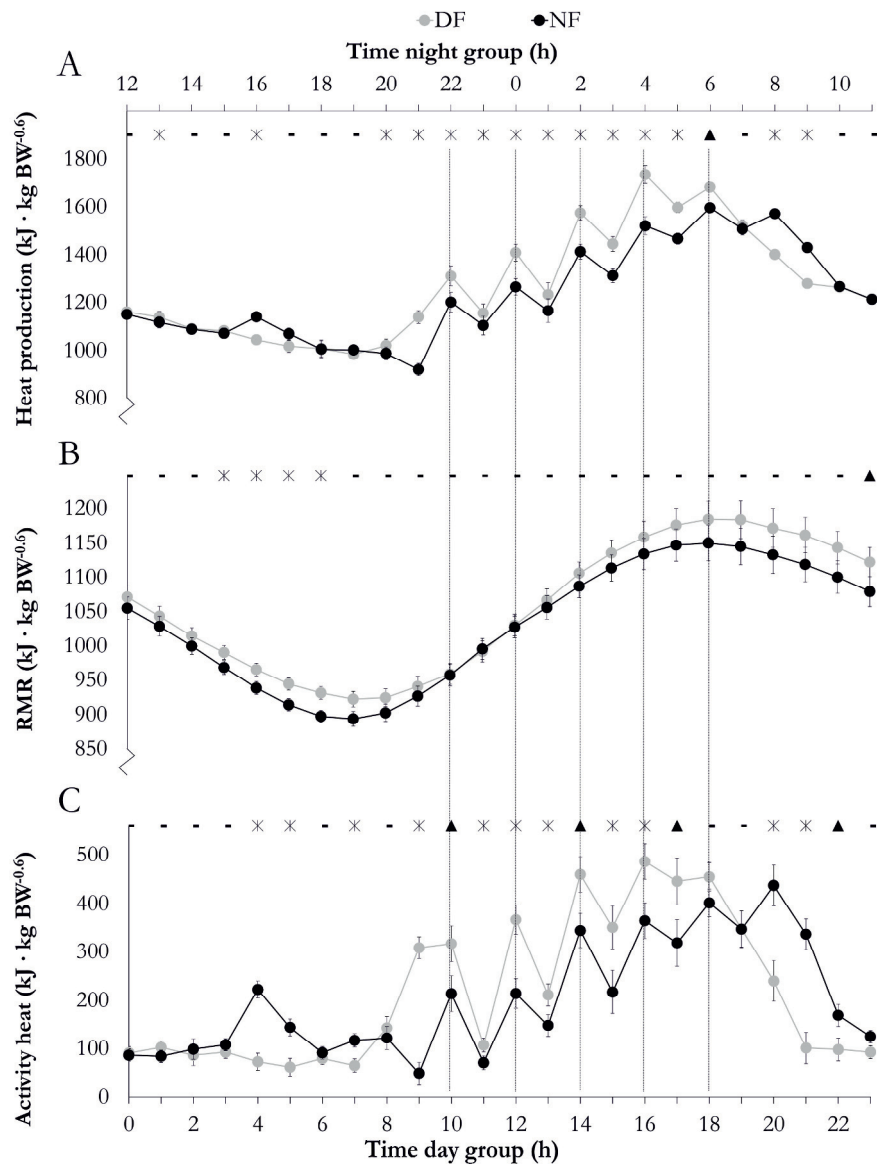
Figure 6.3 Circadian patterns of total heat production (panel A), resting metabolic rate (RMR, panel B), and activity related heat production (panel C) in pigs (BW 42 ±1.8 kg) when feeding five meals bihourly during the day (10.00h – 18.00h; bottom X-axis; Diurnal fed, DF) or night (22.00h – 06.00h; top X-axis; Nocturnal fed, NF). Dotted lines represent feeding times. Values are presented as least square means and pooled standard error of the mean, n=6 with each experimental unit being a group of 5 male pigs. ✳ represents P < 0.05, ▲ represents 0.05 < P < 0.1, - represents P > 0.1.
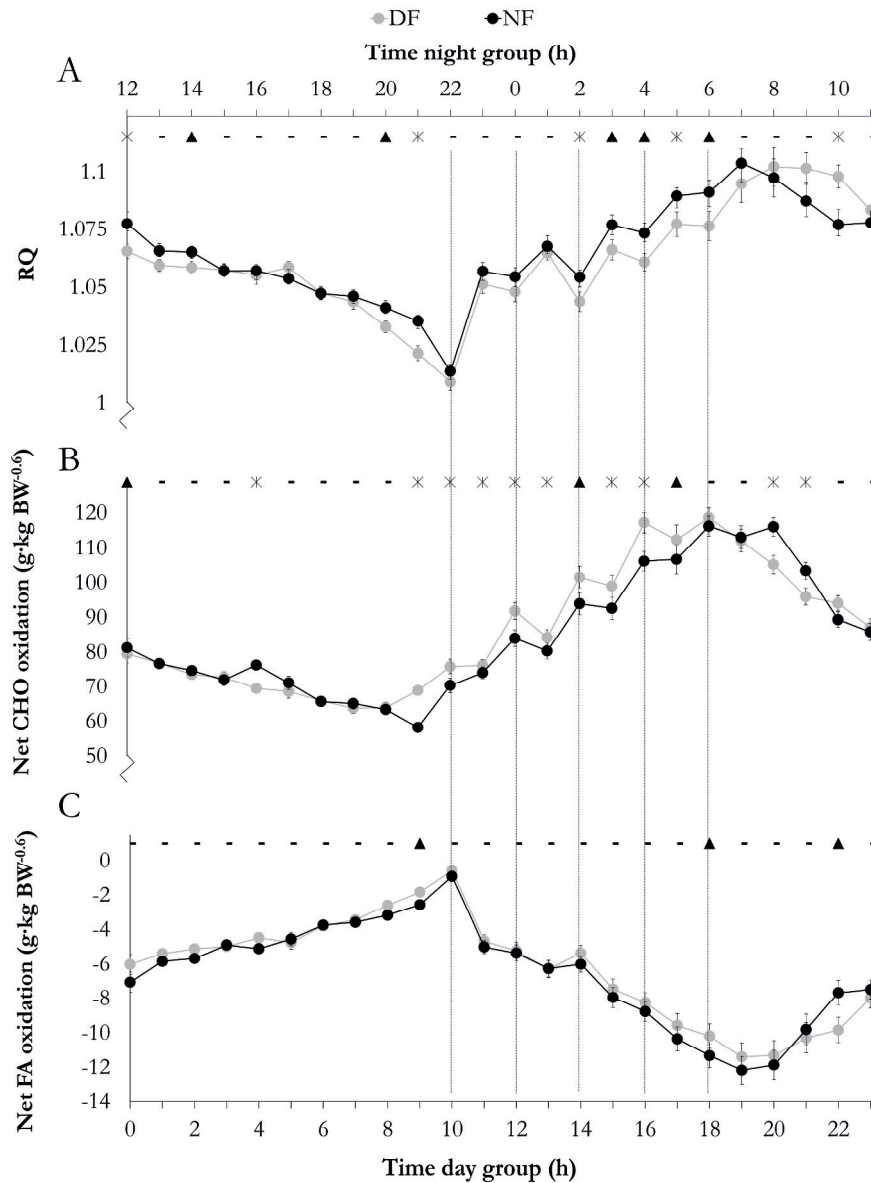
Figure 6.4 Circadian patterns of respiratory quotient (RQ; Panel A), net carbohydrate (CHO) oxidation (Panel B), and net fatty acids (FA) oxidation (panel C) in pigs (BW 42 ±1.8 kg) when feeding five meals bihourly during the day (10.00h – 18.00h; bottom x-axis) or night (22.00h – 06.00h; top x-axis). Dotted lines represent feeding times. Values are presented as least square means and pooled standard error of the mean, n=6, with each experimental unit being a group of 5 male pigs. ✳ represents $P < 0.05$, ▲ represents $0.05 < P < 0.1$, - represents $P > 0.1$.

6.3.3 Heat production and substrate oxidation

Total heat production was greater for DF-pigs than NF-pigs during the feeding period ($P<0.050$; Table 6.2). At one, two, and six hours before the first meal, and two and three hours after the last meal, heat production was smaller for DF-pigs than NF-pigs (Figure 6.3). Activity-related heat production responded to the imposed feeding patterns; DF-pigs were more active than NF-pigs during the feeding period, however, less active three, five, and six hours before the first meal, and three and four hours after the last meal ($P<0.05$). Resting metabolic rate was greater for DF-pigs than NF-pigs during the fasting period reaching statistical significance between 4-8 hours before the first meal ($P<0.050$). On average, RQ did not differ between treatments, however, at the end of the feeding period, between the third and fifth meal, RQ tended to be greater for NF-pigs than DF-pigs (Figure 6.4). For both treatments, RQ exceeded unity continuously, and net FA oxidation remained negative throughout the day. Net CHO oxidation was greater for DF-pigs than NF-pigs and reached significance one hour before the first meal was fed until the last meal was fed.

6.4 Discussion

Disruption of circadian feed intake rhythm in humans, e.g., due to shift work, (social) jet-lag, or night eating syndrome, is associated with metabolic disorders. In our study, circadian misalignment in pigs induced by nocturnal feeding, increased the amount of energy deposited compared with diurnal feeding due to a lower heat production. The surplus of energy in NF-pigs was completely stored as fat confirming our hypothesis that asynchronization of the endogenous circadian clock and the timing of feed intake increases fat deposition in growing pigs. Protein deposition was not affected by nocturnal feeding, which is in contrast with Malmlöf [42] who observed a reduced protein deposition in nocturnal-fed pigs. In the study of Malmlöf [42], however, nocturnal feeding was induced by providing the daily ration in three meals, whereby the third mail was supplied at midnight. In contrast, in our study the complete daily ration was supplied during the night. Our results confirm the association between circadian misalignment and obesity in humans [3-6], and are in line with research in rodents, wherein disruption of the circadian rhythm by diurnal feeding increased body weight [12-14] and abdominal fat [12, 14]. In contrast to these studies, our study controlled for caloric intake and measured complete energy balance, enabling quantification of total fat and protein deposition.

6.4.1 Energy partitioning

When averaged over days, the reduced heat production in NF-pigs was explained by numerical differences in the same direction for activity heat and RMR. Nocturnal-fed pigs were particularly less active than DF-pigs during the feeding period, but were more active

than DF-pigs, particularly 6 h before (16.00h) and 2 h (08.00h) after the feeding period. The increase in activity by NF-pigs at 08.00h coincided with onset of lights; however, no environmental stimulus could explain the increase in activity by NF-pigs at 16.00h. Under *ad libitum* feeding conditions, both activity [43] and feed intake [30] peak in the end of the afternoon, a few hours before the lights are turned off. The increase in activity at 16.00h observed for NF-pigs is therefore most likely imposed by the endogenous central clock, which is entrained by light and regulates the timing of behavioral rhythms [1, 44]. In contrast to NF-pigs, DF-pigs were more active prior to the feeding period and remained more active than NF-pigs after each meal, thereby levelling out the difference in activity heat between treatments in the fasting period. For both treatments, diurnal carbohydrate oxidation coincided with the circadian activity pattern indicating that either free glucose or glucose mobilized from muscle glycogen storages was used as energy source for activity. Resting metabolic rate was numerically lower for NF-pigs than DF-pigs most of the day, starting during the largest meals (4th and 5th meal) and reaching statistical significance in 4 to 7 h before the first meal. The latter is in line with McHill*, et al.* [16] who observed a reduced energy expenditure during scheduled sleep in humans exposed to a night shift schedule. Variation in RMR is explained either by differences in basal metabolic rate, which is associated with maintaining basal life processes, body temperature, and organ functioning [45], or by differences in the thermic effect of feeding (TEF). Because relatively more fat and thus less protein was deposited in NF-pigs than in DF-pigs, basal metabolic rate may have been smaller in NF-pigs due to a lower energy requirement for whole body protein synthesis and breakdown. In addition, in humans, TEF is greater after consumption of a meal in the morning and afternoon than in the evening and night [46, 47] and lower in humans that are exposed to circadian misalignment [46]. These results are in line with the reduced postprandial RMR observed in NF-pigs. Moreover, circadian misalignment induced by shift-work [9], and sleep disturbances [48] is associated with a reduced glucose tolerance resulting in impaired glucose uptake, which in turn is associated with a lower TEF [49-51]. Our results show that both RMR and activity heat reduced total energy expenditure of NF-pigs; hence explain the greater fat deposition in NF-pigs than DF-pigs.

6.4.2 Nutrient digestion

Nocturnal feeding of pigs tended to decrease apparent total tract digestibility (not corrected for endogenous losses) of energy (0.6 %-unit) and DM (0.5 %-unit) compared with diurnal feeding. In humans, circadian misalignment is associated with gastrointestinal disorders related to motility [52], inflammation [53, 54], or cancer [55, 56] indicating that disruption of the circadian clock has a negative impact on gastrointestinal tract health and possibly function in the long term. Short term effects of circadian misalignment on gastro intestinal function are less explicitly investigated. Some digestive processes however, such as secretion

Chapter 6

of gastric acid in humans [57] and pancreatic juice in rats [58], show a clear circadian rhythm independent of feed intake. Consequently, it could be hypothesized that circadian misalignment may negatively affect nutrient digestion. In pigs however, timing of feed intake (08.00h, 16.00h, or 24.00h) did not affect ileal DM and nitrogen digestibility [59] indicating that most likely short-term changes of circadian misalignment on digestive processes in pigs are minor. Nocturnal feeding increased energy losses through methane, a gas mainly produced through microbial fermentation in the large intestine of pigs. Regardless of feeding regime, methane production increased during the feeding period. Possibly increased colonic motor activity, which increases after feed consumption and with physical activity [60, 61], enhanced flatulence, and consequently methane excretion. The greater daily methane production in NF-pigs than DF-pigs indicates that nocturnal feeding has changed the composition and or activity of the microbial population. The composition of the gut microbiome is dynamic as it exhibits diurnal fluctuations, which are influenced by time of eating, as was shown in humans and mice [62, 63]. In mice, these diurnal fluctuations are suppressed by disruption of the host circadian clock thereby changing the fecal microbiota composition [64], which is suggested to influence the host metabolism [19, 65]. For example, transferring intestinal microbiota of jet-lagged humans (circadian misaligned) to germ-free mice, resulted in obesity and glucose intolerance compared with transfer of intestinal microbiota of non-jet-lagged people [63]. These results indicate that the greater fat deposition in NF-pigs than DF-pigs potentially relates to changes in gut microbiota composition, but do not show the relation with methanogens specifically. In humans, a greater methane production is associated with a higher body mass index and fat percentage [66, 67]. It is suggested that methane facilitates and accelerates fermentation through removal of hydrogen atoms [66, 68] consequently increasing microbial SCFAs production and thus nutrient supply for the host. In addition, presence of methane may slow down small intestinal digesta transit [69], thereby increasing nutrient absorption [67]. Consequently, the greater fat deposition in NF-pigs may be related to the greater methane production, however, the causality between circadian misalignment and methane production remains unexplained.

6.5 Conclusion

When maintaining caloric intake and identical meal patterns, nocturnal feeding of pigs reduces heat production, which at equal protein gain resulted in increased fat deposition. In addition, nocturnal feeding altered diurnal activity patterns of pigs, but did not affect the overall level of energy expenditure related to physical activity. This study provides evidence that circadian misalignment has little effect on nutrient digestion, but alters nutrient partitioning, ultimately leading to an increase in fat deposition coinciding with an increase

in methane production. The causality of the association between circadian misalignment and methane remains to be investigated.

Chapter 6

References

1.   Strubbe JH, van Dijk G: The temporal organization of ingestive behaviour and its interaction with regulation of energy balance. *Neurosci Biobehav Rev* 2002, 26:485-498.

2.   Sahar S, Sassone-Corsi P: The epigenetic language of circadian clocks. In *Circadian Clocks*. Edited by Kramer A, Merrow M. London: Springer; 2013: 29-45

3.   Parsons MJ, Moffitt TE, Gregory AM, Goldman-Mellor S, Nolan PM, Poulton R, Caspi A: Social jetlag, obesity and metabolic disorder: investigation in a cohort study. *Int J Obes* 2014, 39:842.

4.   Colles SL, Dixon J, O'brien P: Night eating syndrome and nocturnal snacking: association with obesity, binge eating and psychological distress. *Int J Obes* 2007, 31:1722.

5.   Roenneberg T, Allebrandt KV, Merrow M, Vetter C: Social jetlag and obesity. *Curr Biol* 2012, 22:939-943.

6.   Karlsson B, Knutsson A, Lindahl B: Is there an association between shift work and having a metabolic syndrome? Results from a population based study of 27 485 people. *Occup Environ Med* 2001, 58:747-752.

7.   Leproult R, Holmbäck U, Van Cauter E: Circadian misalignment augments markers of insulin resistance and inflammation, independently of sleep loss. *Diabetes* 2014:DB_131546.

8.   Gan Y, Yang C, Tong X, Sun H, Cong Y, Yin X, Li L, Cao S, Dong X, Gong Y: Shift work and diabetes mellitus: a meta-analysis of observational studies. *Occup Environ Med* 2015, 72:72-78.

9.   Morris CJ, Yang JN, Garcia JI, Myers S, Bozzi I, Wang W, Buxton OM, Shea SA, Scheer FA: Endogenous circadian system and circadian misalignment impact glucose tolerance via separate mechanisms in humans. *Proceedings of the National Academy of Sciences* 2015:201418955.

10.   Morris CJ, Yang JN, Scheer FA: The impact of the circadian timing system on cardiovascular and metabolic function. In *Prog Brain Res. Volume* 199: Elsevier; 2012: 337-358

11.   Ellingsen T, Bener A, Gehani A: Study of shift work and risk of coronary events. *The journal of the Royal Society for the Promotion of Health* 2007, 127:265-267.

12.   Salgado-Delgado R, Angeles-Castellanos M, Saderi N, Buijs RM, Escobar C: Food intake during the normal activity phase prevents obesity and circadian desynchrony in a rat model of night work. *Endocrinology* 2010, 151:1019-1029.

13.   Arble DM, Bass J, Laposky AD, Vitaterna MH, Turek FW: Circadian timing of food intake contributes to weight gain. *Obesity* 2009, 17:2100-2102.

14.   Fonken LK, Workman JL, Walton JC, Weil ZM, Morris JS, Haim A, Nelson RJ: Light at night increases body mass by shifting the time of food intake. *Proceedings of the National Academy of Sciences* 2010, 107:18664-18669.

15.   Goel N, Stunkard AJ, Rogers NL, Van Dongen HP, Allison KC, O'Reardon JP, Ahima RS, Cummings DE, Heo M, Dinges DF: Circadian rhythm profiles in women with night eating syndrome. *J Biol Rhythms* 2009, 24:85-94.

16.   McHill AW, Melanson EL, Higgins J, Connick E, Moehlman TM, Stothard ER, Wright KP: Impact of circadian misalignment on energy metabolism during simulated nightshift work. *Proceedings of the National Academy of Sciences* 2014, 111:17302-17307.

17.   Bray MS, Young ME: Circadian rhythms in the development of obesity: potential role for the circadian clock within the adipocyte. *Obes Rev* 2007, 8:169-181.

18. Westerterp-Plantenga M, Ijedema M, Wijckmans-Duijsens N: The role of macronutrient selection in determining patterns of food intake in obese and non-obese women. *Eur J Clin Nutr* 1996, 50:580-591.

19. Asher G, Sassone-Corsi P: Time for food: the intimate interplay between nutrition, metabolism, and the circadian clock. *Cell* 2015, 161:84-92.

20. Roura E, Koopmans S-J, Lallès J-P, Le Huerou-Luron I, De Jager N, Schuurman T, Val-Laillet D: Critical review evaluating the pig as a model for human nutritional physiology. *Nutrition research reviews* 2016, 29:60-90.

21. Baker DH: Animal models in nutrition research. *J Nutr* 2008, 138:391-396.

22. Quiniou N, Dubois S, Noblet J: Voluntary feed intake and feeding behaviour of group-housed growing pigs are affected by ambient temperature and body weight. *Livest Prod Sci* 2000, 63:245-253.

23. Feddes J, Young B, DeShazer J: Influence of temperature and light on feeding behaviour of pigs. *Appl Anim Behav Sci* 1989, 23:215-222.

24. Nielsen BL, Lawrence AB, Whittemore CT: Effect of group size on feeding behaviour, social behaviour, and performance of growing pigs using single-space feeders. *Livest Prod Sci* 1995, 44:73-85.

25. Geverink N, Schouten W, Gort G, Wiegant V: Individual differences in behaviour, physiology and pathology in breeding gilts housed in groups or stalls. *Appl Anim Behav Sci* 2003, 81:29-41.

26. Boumans IJ, de Boer IJ, Hofstede GJ, la Fleur SE, Bokkers EA: The importance of hormonal circadian rhythms in daily feeding patterns: An illustration with simulated pigs. *Horm Behav* 2017, 93:82-93.

27. Heetkamp MJW, Alferink SJJ, Zandstra T, Hendriks P, Brand Hvd, Gerrits WJJ: Chapter 2: Design of climate respiration chambers, adjustable to the metabolic mass of subjects. In *Indirect calorimetry*. 2015: 35-56

28. Centraal Veevoeder Bureau: *CVB table pigs*. The Hague2012.

29. Noblet J, Karege C, Dubois S, Van Milgen J: Metabolic utilization of energy and maintenance requirements in growing pigs: effects of sex and genotype. *J Anim Sci* 1999, 77:1208-1216.

30. De Haer L, Merks J: Patterns of daily food intake in growing pigs. *Anim Sci* 1992, 54:95-104.

31. Gundlach H: Verhaltensontogenese und tagesperiodik beim europaishen wildaischwein (Sus scrofa L). *Zeitschrift Tierpsychologie* 1968, 25:955-995.

32. D'Eath RB, Turner SP: The natural behaviour of the pig. In *The welfare of pigs*. Springer; 2009: 13-45

33. Heetkamp MJW, Alferink SJJ, Zandstra T, Hendriks P, Brand Hvd, Gerrits WJJ: Design of climate respiration chambers, adjustable to the metabolic mass of subjects. In *Indirect calorimetry*. Edited by Gerrits WJJ, Labussière E. Wageningen: Wageningen Academic Publishers; 2015: 35-56

34. ISO 6496: *ISO 6496: Animal feeding stuffs - Determination of moisture and other volatile matter content*. Geneva, Switzerland: International Organization for Standardization; 1999.

35. ISO 9831: *ISO 9831: Animal feeding stuff - Determination of gross calorific value - Bomb calorimetry method*. Geneva, Switzerland: International Organization for Standardization; 1998.

36. ISO 5983: *ISO 5983: Animal feeding stuffs - Determination of N content and calculation of rude protein content - Part 1: Kjeldahl method*. Geneva, Switzerland: International Organization for Standardization; 2005.

37. Myers WD, Ludden PA, Nayigihugu V, Hess BW: A procedure for the preparation and quantitative analysis of samples for titanium dioxide. *Journal of Animal Science* 2004, 82:179-183.

Chapter 6

38. Brouwer E: Report of sub-committee on constants and factors. In *Energy metabolism*. Edited by Blaxter KL: Londen-New York: Academic Press; 1965: 442-443

39. Van den Borne JJGC, Heetkamp MJW, Alferink SJJ, Gerrits WJJ: Moving from a complete energy balance towards substrate oxidation: use of stable isotopes In *Indirect calorimetry*. Edited by Gerrits WJJ, Labussière E. Wageningen: Wageningen Academic Publishers; 2015: 87-113

40. Van Klinken JB, van den Berg SA, Havekes LM, Van Dijk KW: Estimation of activity related energy expenditure and resting metabolic rate in freely moving mice from indirect calorimetry data. *PLoS One* 2012, 7:e36162.

41. Kotb A, Luckey T: Markers in nutrition. In *Nutr Abstr Rev*1972: 813-845.

42. Malmlöf K: Metabolic responses to nocturnal feeding in the growing pig. *J Anim Physiol Anim Nutr (Berl)* 1986, 56:96-103.

43. van Erp RJJ, van Hees HMJ, Zijlstra RT, van Kempen TATG, van Klinken JB, Gerrits WJJ: Reduced feed intake, rather than increased energy losses, explains variation in growth rates of normal-birth-weight piglets. *J Nutr* 2018, 148:1794-1803.

44. Johnston JD: Physiological responses to food intake throughout the day. *Nutrition research reviews* 2014, 27:107-118.

45. Kuhla B, Derno M, Metges CC: Indirect calorimetry for elucidating dynamics in energy metabolism of farm animals. In *Indirect calorimetry*. Edited by Gerrits WJJ, Labussière E. Wageningen: Wageningen Academic Publishers; 2015: 115-133

46. Morris CJ, Garcia JI, Myers S, Yang JN, Trienekens N, Scheer FA: The human circadian system has a dominating role in causing the morning/evening difference in diet-induced thermogenesis. *Obesity* 2015, 23:2053-2058.

47. Romon M, Edme J-L, Boulenguez C, Lescroart J-L, Frimat P: Circadian variation of diet-induced thermogenesis. *Am J Clin Nutr* 1993, 57:476-480.

48. Nedeltcheva AV, Scheer FA: Metabolic effects of sleep disruption, links to obesity and diabetes. *Curr Opin Endocrinol Diabetes Obes* 2014, 21:293.

49. Ravussin E, Acheson K, Vernet O, Danforth E, Jequier E: Evidence that insulin resistance is responsible for the decreased thermic effect of glucose in human obesity. *J Clin Invest* 1985, 76:1268-1273.

50. Ravussin E, Bogardus C, Schwartz R, Robbins D, Wolfe R, Horton E, Danforth E, Sims E: Thermic effect of infused glucose and insulin in man. Decreased response with increased insulin resistance in obesity and noninsulin-dependent diabetes mellitus. *J Clin Invest* 1983, 72:893-902.

51. Segal KR, Albu J, Chun A, Edano A, Legaspi B, Pi-Sunyer F: Independent effects of obesity and insulin resistance on postprandial thermogenesis in men. *J Clin Invest* 1992, 89:824-833.

52. Lu WZ, Gwee KA, Ho KY: Functional bowel disorders in rotating shift nurses may be related to sleep disturbances. *Eur J Gastroenterol Hepatol* 2006, 18:623-627.

53. Scheving LA, Russell WE: It's about time: clock genes unveiled in the gut. *Gastroenterology* 2007, 133:1373-1376.

54. Bron R, Furness JB: Rhythm of digestion: keeping time in the gastrointestinal tract. *Clin Exp Pharmacol Physiol* 2009, 36:1041-1048.

55. Schernhammer ES, Laden F, Speizer FE, Willett WC, Hunter DJ, Kawachi I, Fuchs CS, Colditz GA: Night-shift work and risk of colorectal cancer in the nurses' health study. *J Natl Cancer Inst* 2003, 95:825-828.

56.   Haus E, Smolensky M: Biological clocks and shift work: circadian dysregulation and potential long-term effects. *Cancer Causes Control* 2006, 17:489-500.

57.   Moore J, Englert E: Circadian rhythm of gastric acid secretion in man. *Nature* 1970, 226:1261.

58.   Maouyo D, Sarfati P, Guan D, Morisset J, Adelson JW: Circadian rhythm of exocrine pancreatic secretion in rats: major and minor cycles. *Am J Physiol Gastrointest Liver Physiol* 1993, 264:G792-G800.

59.   Jørgensen H, Lindberg JE, Andersson C: Diurnal variation in the composition of ileal digesta and the ileal digestibilities of nutrients in growing pigs. *J Sci Food Agric* 1997, 74:244-250.

60.   Narducci F, Bassotti G, Gaburri M, Morelli A: Twenty four hour manometric recording of colonic motor activity in healthy man. *Gut* 1987, 28:17-25.

61.   Frexinos J, Bueno L, Fioramonti J: Diurnal changes in myoelectric spiking activity of the human colon. *Gastroenterology* 1985, 88:1104-1110.

62.   Zarrinpar A, Chaix A, Yooseph S, Panda S: Diet and feeding pattern affect the diurnal dynamics of the gut microbiome. *Cell metabolism* 2014, 20:1006-1017.

63.   Thaiss CA, Zeevi D, Levy M, Zilberman-Schapira G, Suez J, Tengeler AC, Abramson L, Katz MN, Korem T, Zmora N: Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. *Cell* 2014, 159:514-529.

64.   Liang X, Bushman FD, FitzGerald GA: Rhythmicity of the intestinal microbiota is regulated by gender and the host circadian clock. *Proceedings of the National Academy of Sciences* 2015, 112:10479-10484.

65.   Voigt R, Forsyth C, Green S, Engen P, Keshavarzian A: Circadian rhythm and the gut microbiome. In *Int Rev Neurobiol. Volume* 131: Elsevier; 2016: 193-205

66.   Basseri RJ, Basseri B, Pimentel M, Chong K, Youdim A, Low K, Hwang L, Soffer E, Chang C, Mathur R: Intestinal methane production in obese individuals is associated with a higher body mass index. *Gastroenterology & hepatology* 2012, 8:22.

67.   Mathur R, Amichai M, Chua K, Mirocha J, Barlow G, Pimentel M: Methane and hydrogen positivity on breath test is associated with greater body mass index and body fat. *J Clin Endocrinol Metab* 2013, 98:E698-E702.

68.   Schink B: Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol Rev* 1997, 61:262-280.

69.   Pimentel M, Lin HC, Enayati P, van den Burg B, Lee H-R, Chen JH, Park S, Kong Y, Conklin J: Methane, a gas produced by enteric bacteria, slows intestinal transit and augments small intestinal contractile activity. *Am J Physiol Gastrointest Liver Physiol* 2006, 290:G1089-G1095.

Chapter 6

Chapter 7

General Discussion

7.1 Introduction

It is commonly assumed that starch fermented by bacteria yields less energy than starch digested by host-enzymes, and thus potentially compromises growth rates of pigs. Besides the proportion of undigested starch, i.e., resistant starch (RS), affected by the extent of starch digestion, also the rate of starch digestion is suggested to affect pig performance through its effect on postprandial blood glucose concentration and insulin response [1]. A slow or moderate postprandial insulin release is suggested to be beneficial for muscle growth, whereas increased and prolonged postprandial insulin concentrations may stimulate fat deposition [2]. However, literature on the effect of starch digestion kinetics on pig performance is inconclusive (Chapter 1). Therefore, this thesis aimed to create a better understanding of underlying processes that are affected by starch digestion kinetics to determine its effect on pig performance more accurately. In Chapter 2 and 3 of this thesis, the effect of the extent and rate of starch digestion on growth performance of pigs were investigated. To get a better prediction of total starch fermented, and thus the extent of its enzymatic digestion, we quantified small intestinal starch fermentation in Chapter 4. It was suggested that differences in meal patterns and feeding regime may explain the contradictory findings of RS on growth performance in literature; a smaller number of large meals results in a greater increase in postprandial glucose concentrations than frequent intake of small meals. Therefore, we investigated both long-term (Chapter 2) and short-term (Chapter 5) effects of RS on meal patterns under *ad libitum* conditions. In these studies, we observed that the daily feed intake pattern of pigs follows a clear circadian rhythm regardless of diet or treatment. In Chapter 6, we investigated the importance of shifts in this pattern by measuring energy utilization in pigs that were forced to deviate from their "normal" feeding rhythm. In this chapter I will discuss the impact of starch digestion kinetics on pigs by combining results of this thesis with existing literature. In the first part of this chapter, I will discuss the importance of both the rate and extent of starch digestion for growth performance in pigs, and the extent in which starch is fermented proximal to the large intestine. In the second part, I will discuss the difference in energy utilization between digested and fermented starch on the basis of current net energy (NE) systems. Finally, the impact of feeding strategies on meal patterns will be discussed, particularly with regards to resistant starch. I will close this chapter by presenting my main conclusions and implications of my results.

7.2 Starch digestion kinetics and pig performance

7.2.1 Extent versus rate of starch digestion

In Chapter 2 we showed that total starch disappearance *in vivo* was lower in the proximal (36% compared with 41%), mid (69% compared with 85%), and distal (74% compared with 98%) small intestine of pigs fed a diet containing high-amylose starch (HRS) than pigs fed a diet containing waxy maize starch (LRS), whereas the mean retention time of digesta in the small intestine was the same (~120 min). Because a greater part of the starch in HRS (26%) than in LRS (2%) escaped small intestinal digestion, the rate of starch digestion should be expressed as a proportion of the total small intestinal starch disappearance when comparing the two diets. When expressing the results of Chapter 2 in this way, the amount of starch digested after ~10 min (proximal SI, 45% of total starch digested) and ~55 min (mid SI, 90% of total starch digested) in the small intestine were similar for HRS-fed and LRS-fed pigs. These results are in line with Martens*, et al.* [3] from which can be calculated that 36% (~4 min), 53% (~20 min), and 91% (~55 min) high amylose maize starch disappeared from the proximal, mid, and distal small intestine when expressed as a proportion of total starch disappearance in the small intestine (55%; ~110 min). In contrast to the study of Martens*, et al.* [3], diets used in Chapter 2 were pelleted, which may gelatinized part of the starch [4] increasing its total digestion [5], but apparently not its rate (% digestible starch digested/min). These calculations indicate that the time postprandial blood glucose levels required to peak, which is affected by the rate of starch digestion (Chapter 1), did not differ between diets used in Chapter 2. In contrast, the magnitude of the postprandial glucose peak was lower in HRS-fed pigs than LRS-fed pigs due to a lower extent of starch digestion. Simultaneously, SCFA supply increased by microbial fermentation of undigested starch in HRS-fed pigs, but not in LRS-fed pigs. These changes in nutrient supply with RS intake did not affect growth rates, and fat and protein deposition in pigs that had *ad libitum* access to feed (Chapter 2). The latter was substantiated by a similar back-fat thickness, meat percentage, and efficiency to use feed for carcass gain observed in pigs fed high and low RS diets. In contrast to the diets used in Chapter 2, both diets fed in Chapter 3 were assumed to be primarily digested in the small intestine, but to differ in their rate of digestion [6]. Consequently, the total dietary glucose uptake was similar between diets, but postprandial blood glucose levels peaked faster with rapidly digestible rice starch (RDS) than with slowly digestible pea starch (SDS). RDS increased fat deposition when compared to SDS (~ 2% more fat per % extra GE intake), which became apparent at greater feeding levels when postprandial *de novo* fatty acid synthesis is continuously stimulated and less body fat is oxidized (RQ>1; Chapter 3). Hence, the rate of starch digestion changed the fate of dietary glucose and herewith also body fat deposition in pigs. The contrast in findings on

Chapter 7

performance of pigs between Chapter 2 and 3 emphasizes the importance to make a distinction between the rate and extent of starch digestion. Apparently the load of glucose, determined by the extent of starch digestion, has a lower impact on pig performance than the rate in which glucose becomes available. It should be noted though, that a lower load of glucose as a consequence of a lower extent of starch digestion, is confounded with an increase in dietary RS concentration; virtually all starch escaping digestion in the small intestine is fermented in the large intestine (Chapter 2,4 and others [7, 8]). Effects related to RS on nutrient digestion and utilization should always be considered when studying the effect of the extent of starch digestion on pig performance.

7.2.2 Overestimating starch digestion?

In Chapter 5 we reported that the maximum extent of *in vitro* starch digestion of the waxy maize starch diet (99.9%) was reached at approximately 150 minutes and that of the high-amylose maize starch diet (63.4%) at approximately 200 minutes. In contrast, *in vivo*, 98% dietary waxy maize starch and 74% dietary high-amylose starch disappeared from the small intestine after a residence time of 120 minutes (Chapter 2). These results indicate that starch is either more efficiently digested *in vivo* than *in vitro* or underestimated *in vitro* due to its methodological limitations [3]; e.g., by absence of brush border enzymes [9, 10]. The gap between *in vitro* and *in vivo* may also be explained by hydrolysis of starch in the stomach not accounted for *in vitro* [11] or by errors in the measurement of digesta passage kinetics [3]. The latter, however, would only affect the rate of starch digestion, and not its extent. Alternatively, part of the starch that disappeared from the small intestine may be fermented by bacteria in the upper gastrointestinal tract. In addition, a gap exists between digested glucose and glucose appearing in the portal vein, ranging between 8 to 26% [6, 12-14]. This is often explained by intestinal utilization of glucose, but microbial fermentation may also contribute to this. Noah*, et al.* [15] suggested this to be of minor importance, as SCFA concentrations in small intestinal digesta were not increased in their study. However, intestinal SCFA digesta concentrations are affected by both the microbial SCFA production and the rate of SCFA absorption, and therefore its concentration in the gut not necessarily reflects the quantity of starch fermented. Although we were not able to quantify ileal starch fermentation directly using the ^{13}C-based method described by Gerrits*, et al.* [7] (Chapter 4), results reported in Chapter 4 and 5 showed that starch fermentation in the upper gastrointestinal tract may be substantial. Even in pigs fed digestible starch (waxy maize starch; ileal starch disappearance, 98%), ~10% of total starch intake was estimated to be fermented. In addition, in Chapter 5 we showed that enzymatically digestible starch may partly be fermented in growing pigs when fermentable starch is substituted for digestible starch. This was either explained by a more rapid and consequently increased flow of digestible starch into the large intestine or explained by increased competition between

enzymes and bacteria for substrate in the small intestine. Mean retention time in the small intestine was not affected by starch source in Chapter 2 (high amylose maize starch, 2.0 h; waxy maize starch, 2.1 h) indicating that this phenomenon was most likely explained by small intestinal fermentation. Results reported in this thesis suggest that starch digestion based on ileal digestibility values overestimates starch digestion by host-enzymes by 1-7 % when not considering ileal starch fermentation.

7.2.3 Points of concern: Starch digestion kinetics and pig performance

In Chapter 2 we observed that the extent of starch digestion, i.e., the amount of starch that is digested and fermented, did not affect growth performance of pigs. In contrast to our results, others reported either improved growth rates and feed efficiencies [1, 16] or reduced growth rates in RS-fed pigs [8, 17]. Growth rates and feed efficiencies are often based on live weight. Consequently, performance of pigs fed high RS diets may be overestimated when not corrected for the increase in intestinal weight (Chapter 2 and others [18, 19]). Reduced growth rates are often observed in RS-fed weaned piglets and explained by a reduced feed intake [8, 17]. Fouhse, *et al.* [8] showed that 21 days after weaning, virtually all starch completely disappears in the large intestine of pigs, regardless of the RS concentration in the diet fed. These results indicate that starch fermentation is not limiting the energy intake of the pig at this age. However, the fermentation capacity of a newly-weaned piglet is still increasing [20]. As a result, fermentation processes in newly-weaned piglets possibly require more time to adapt to RS intake than in older pigs. In addition, the capacity to digest starch also increases with age (Chapter 5), which is explained by increasing carbohydrase activity [21-24]. Consequently, young pigs may be less capable to digest high RS diets than older pigs. In Chapter 5, we concluded that inadequate adaptation to increasing dietary RS concentration reduces feed intake in growing pigs (BW trajectory: 30 – 60 kg). Inadequate adaptation of fermentative and digestive processes to RS intake may as well explain the reduced feed intake, and consequently lower growth performance of piglets after weaning. Future studies should take both the increasing effect of RS on intestinal weight and the age of the pig into consideration when investigating the effect of RS on growth performance of pigs.

7.3 Digested or fermented starch, does it matter?

In current feed evaluation systems, the net energy (NE) value of feed ingredients for growing pigs is defined as its energetic contribution to maintenance and growth, i.e., energy retention (ER). The conversion of gross energy (GE; i.e., enthalpy value of a feed ingredient) to NE can be calculated in three steps (Figure 7.1). (1) Fecal energy losses are subtracted from GE intake to calculate digestible energy (DE). (2) Energy losses with urine and gas

production through microbial fermentation are subtracted from DE to calculate metabolizable energy (ME). (3) Heat losses are subtracted from ME to calculate NE. Finally, NE can be used for maintenance or ER. For the calculations of NE values of feed ingredients, it is assumed that the efficiency to use energy for maintenance is similar to that of energy used for ER and that maintenance energy is a constant. Hence, NE values are incremental; i.e., differences between NE values of feed ingredients are explained by differences in the marginal efficiency in which energy is used for ER.
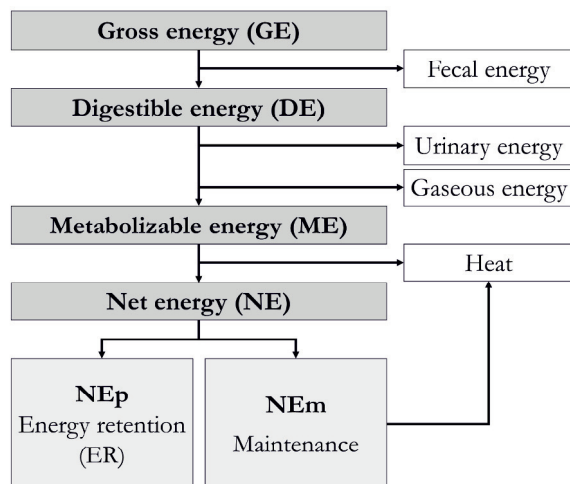
Figure 7.1 Principle of energy evaluation and energy requirements in growing pigs. Adapted from Blok, *et al.* [25].

NE values of feed ingredients are calculated using prediction equations, which have been generated from large datasets containing energy and nutrient digestibility values. These values are often measured in individually housed, meal-fed pigs fed diets varying in ingredient composition. These prediction equations differ between feed evaluation systems. E.g., the National Research Council (NRC) starts from a measured DE value of feed ingredients, and applies a NE:DE ratio that is dependent on the fat, starch, crude protein, and acid detergent fiber content [26]. Centraal Veevoeder Bureau (CVB) uses measured values for the apparent total tract digestible content of fat, protein, and starch + sugars and fermentable nutrients (RS, non-starch polysaccharides (NSP), fermentable sugars) for each feed ingredient and applies partial energetic efficiencies for each digestible nutrient to calculate its NE value [25]. Although it is acknowledged by most feed evaluation systems that all fermented carbohydrates negatively affect NE when substituted for digestible carbohydrates, only CVB has included RS in their NE calculation. CVB corrects total starch content for its RS fraction to calculate the digestible starch content, and assumes that the RS fraction yields the same amount of energy as other fermented carbohydrates, which is

30% lower than that of digestible carbohydrates [25]. The difference in NE value is explained by greater energy losses related to the fermentation and utilization of undigested carbohydrates (Figure 7.2). In Chapter 2, however, growth performance of pigs fed RS appeared to be similar to that of pigs fed digestible starch suggesting that the net energy value of RS currently used in pig feed formulation is underestimated. This will further be discussed in the next paragraphs.
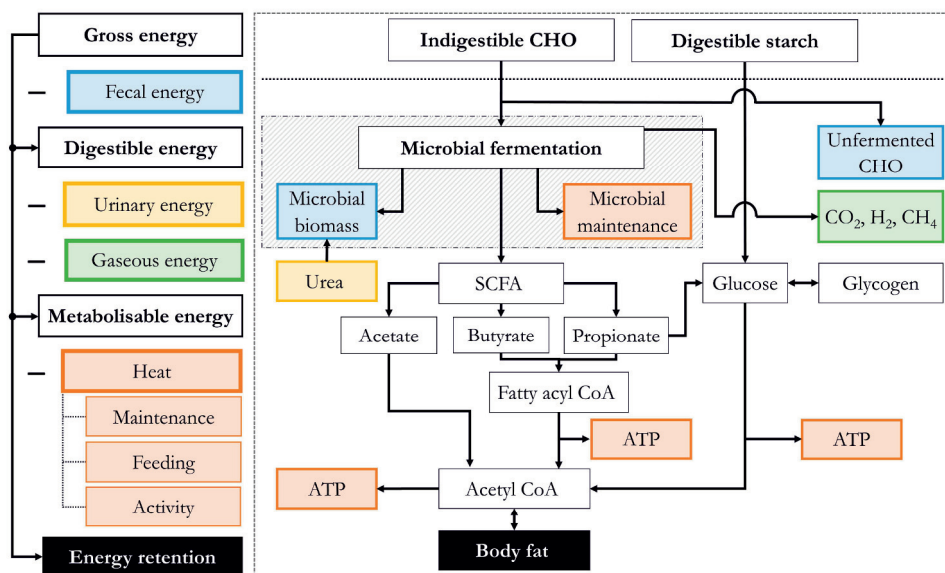
Figure 7.2 Schematic overview of the utilization of digestible starch and indigestible carbohydrates and its effect on energy balance. Colored pools in flow diagram (right-side) indicate the effect of this pool on specific energy losses in the energy balance (left-side; blue: fecal energy, yellow: urinary energy, green: gaseous energy, and orange: heat). ATP, Adenosine triphosphate; CHO, carbohydrates, DE, digestible energy; GE, gross energy; ME, metabolizable energy; NE, net energy; SCFA, short-chain fatty acids.

7.3.1 Utilization of digested versus fermented energy

Digested and fermented energy from carbohydrates

Both fermented and digested carbohydrates have a gross energy (GE) value of approximately 17.5 kJ/g [27]. The efficiency in which this energy is utilized for growth varies depending on the energy losses occurring during digestion, fermentation and post-absorptive metabolism. All digestible carbohydrates are assumed to be digested into glucose or other monosaccharides in the small intestine, which are subsequently absorbed and utilized by the pig. Undigested carbohydrates serve as a substrate for microbial fermentation. Only part of the undigested carbohydrates is fermented, depending, amongst others, on the degree of lignification and residence time at the site of fermentation. Consequently, part of

the difference in energy gain between pigs fed digestible and indigestible carbohydrates can be explained by energy losses through fecal excretion of unfermented material. Using DE as a basis for NE calculations instead of GE takes this difference into account. Ignoring fermentation in the small intestine, several researchers have compared the efficiency in which digested and fermented energy are utilized for NE. Digested energy equals the proportion of DE that is absorbed in upper gastro intestinal tract, whereas fermented energy is calculated as the difference between total tract energy disappearance and digested energy, hence equals the proportion of DE that is absorbed in the hind-gut. The efficiency to utilize 1 additional %-unit DE absorbed from the hindgut for NE is on average 30% lower than when this same proportion is absorbed from the small intestine [28-30]. This value was estimated either as the marginal efficiency in which fermented energy is used for NE by replacing dietary starch for dietary indigestible but fermentable carbohydrates [28, 29], or by regression of energy digested and fermented on ER (Figure 7.3) using a large dataset containing 61 diets varying in the proportion of fermented energy (min: 2.9% of DE; max 26.9% of DE) [30].
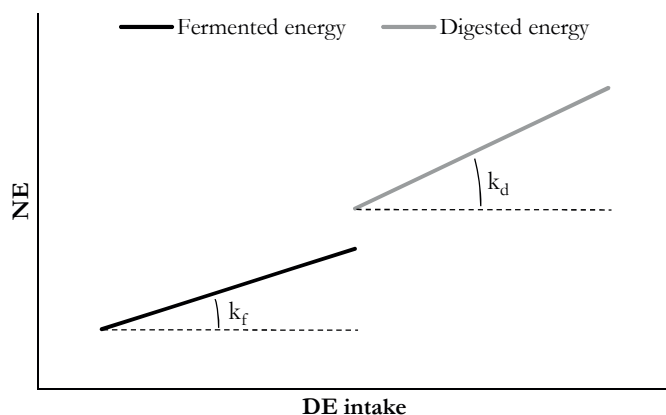
Figure 7.3 Estimation of the efficiency in which digested energy and fermented energy are utilized for NE using the regression model: NE = k_d × digested energy + k_f × fermented energy [30]. The slope of each line represents the efficiency in which digested (k_d) and fermented energy (k_f) are utilized for NE.

Resistant starch completely disappears in the gastrointestinal tract of pigs (Chapter 2, 4, and 5), therefore, its DE value equals its GE value, which in turn equals that of digestible starch. Consequently, GE efficiencies of RS and digestible starch can directly be compared. Gerrits, *et al.* [7] showed that the marginal GE efficiency for NE was 17% lower for RS than for digested starch; hence, per 100 kJ extra GE, 17 kJ less energy is retained when this energy (GE) is derived from RS than when derived from digestible starch. From the data of Gerrits, *et al.* [7] can be calculated that the marginal DE efficiency for ER is only 4% lower for RS than for digested starch; hence, per 100 kJ extra DE, only 4 kJ less energy is retained when

this energy (DE) is derived from RS than when derived from digestible starch. The difference between the marginal GE and DE efficiency of RS is explained by an increased fecal energy output (and thus reduced DE content) when feeding RS; per 100 kJ extra GE, 47 kJ less DE is available when GE is derived from RS instead of digestible starch. The difference in marginal DE efficiency between starch and RS is much lower than the difference of 30% in marginal efficiency between digested and fermented carbohydrates reported by CVB [25]. This may indicate that the efficiency to utilize fermented carbohydrates may not be fixed as currently assumed, but depends on the substrate fermented.

Factors affecting the proportion of digested and fermented energy

Interactions between nutrients or ingredients, occurring inside the digestive tract, influence estimates of digested and fermented energy. For example, dietary fiber may negatively affect the digestion of other nutrients [31, 32]. Consequently, the supply of, and ratio between absorbed nutrients changes, thereby influencing NE efficiency as starch, protein, and fat are each metabolized with a different efficiency [25, 30, 33]. In addition, microbial fermentation stimulates biomass synthesis, shifting nitrogen excretion from urine to feces [7, 31, 34, 35] increasing fecal energy losses, and consequently decreasing DE content. This is mainly explained by incorporation of ammonia-N in microbial biomass reducing the absorption of ammonia by the large intestine into the blood [36]. When enough fermentative substrate is available, urea is secreted from the blood into the large intestine, which is subsequently incorporated in the microbial biomass as well [12]. Because part of the increase in fecal energy with high fermentable diets coincides with a shift in nitrogen excretion from urine to feces, it is impossible to accurately estimate the amount of undigested dietary energy, and thus the proportion of fermented energy, solely from fecal output. In addition, the magnitude of the shift of urinary to fecal energy losses may vary as urinary energy losses decrease with incremental intake of cellulose [28] and retrograded maize starch [7], but not with pea fibers [29]. Heijnen and Beynen [34] showed that retrograded RS decreased urinary nitrogen excretion, whereas uncooked RS did not, when exchanged based on *in vitro* RS content. These results indicate that the shift from urinary energy losses to fecal energy losses is affected by the substrate fermented, possibly due to differences in fermentation kinetics affecting the site and extent of fermentation or due to differences in growth efficiency between bacteria. From data of Gerrits, *et al.* [7], it can be calculated that the decrease in urinary energy losses ($7.62 \cdot kJ\ BW^{0.6}$) only explains 20% of the difference in urinary energy losses between pigs fed RS and digestible starch ($38\ kJ \cdot kg\ BW^{0.6}$), assuming that this difference is completely explained by a lower urinary urea excretion (enthalpy value Urea: 628 kJ/mole; [37]). It is yet unclear what non-nitrogen, energy containing components are not excreted through urine with high RS diets but are with low RS diets explaining the 80%

Chapter 7

unaccounted for by urea. Finally, ignoring fermentation proximal to the large intestine may result in an underestimation of the proportion of fermented energy as well. Inaccuracies in the proportioning of DE to digested and fermented energy results in an incorrect prediction in the efficiency in which these proportions are used for NE. Inaccurate estimates of the amount of digested protein and starch have a similar effect on the prediction of NE values that are estimated by regression of digested and fermented nutrients on NE [25]. Digested protein and starch are both included in this model, but are also used to calculate fermented NSP, which in turn is included in the fraction of fermentable carbohydrates. Because the NE values of nutrients are estimated in the same regression model, inaccurate predictions of one of these nutrient fractions will affect the NE value of all the other nutrients. Consequently, this results in incorrect NE values of feed ingredients stressing out the importance for accurate estimates of digested and fermented nutrients or energy.

7.3.2 Variation in microbial efficiency

The primary function of microbial fermentation is the release of energy through ATP production that can be used by bacteria either for their maintenance or for microbial biomass synthesis. Part of the GE value of fermentable carbohydrates is still captured and released in the form of SCFAs (mainly acetate, propionate, and butyrate) that are available for absorption for the host. Another part is lost through heat and through gaseous production of methane, hydrogen, and carbon dioxide that are produced during the process of fermentation.

Fermentation heat

Based on results of stoichiometric calculations in ruminants [38], fermentation heat represents about 6% of fermented energy. When indirect calorimetry is used to estimate NE efficiency of fermented energy, fermentation heat contributes to the total heat loss of a pig. However, it can be hypothesized that in some conditions fermentation heat is not an additive loss of heat for the host, but is used by the host to warm up its body thereby saving nutrients that otherwise would have been used to maintain body temperature. Subsequently, saved nutrients can serve a different metabolic purpose, e.g., growth. This may specifically be of interest for young animals for which critical temperatures are higher than for older animals.

Gaseous energy losses

Methanogenic archaea use hydrogen to reduce carbon dioxide into methane. On average, the production of methane in growing pigs represents 3 to 7 % of the energy of fermentable carbohydrates [29, 39, 40]. In comparison to methane, hydrogen excretion is low; *in vitro* incubation of high fiber diets with pig inoculum showed that production of hydrogen was on average 40% of that of methane [41]. In addition, hydrogen (10.8 MJ/L) generates

approximately 3 times less energy per L than methane (34.8 MJ/L), and therefore hydrogen losses accounts for less than 1% of the energy from fermentable carbohydrates. Because RS does not stimulate methane production (Chapter 5 and [7, 35, 42]), energy losses related to microbial fermentation may be slightly lower for RS than for other carbohydrates. Methanogens prefer a neutral or slightly alkaline environment [43]. Because pH in the proximal large intestine (chapter 2: caecum, 5.5; proximal colon, 6) is lower than in the distal large intestine (Chapter 2: distal colon, 6.5), the abundance of methanogens is lower in the proximal part [41]; the part in which RS is primarily fermented [44]. Because acetogenesis favors acidic conditions [43], reductive acetogenesis instead of methanogenesis may have been used as hydrogen sink increasing the production of acetate, and thus DE intake.

SCFA production

The amount of SCFAs produced and microbial biomass formed per g of substrate are affected by its fermentation kinetics and by the growth efficiency of the bacteria involved. It is often assumed that substrates that are rapidly fermented have a higher NE value because they deliver more SCFAs for the host, but when expressed per g of substrate fermented this may not be true. When a substrate is slowly fermented less energy is available for bacterial growth, because maintenance requirements must be met in preference to growth. In contrast, when a substrate is rapidly fermented the proportion of energy that can be used for microbial biomass synthesis increases, thereby decreasing the proportion of energy that is released as SCFAs. Consequently, *in vitro* incubation of slowly fermented cellulose with isolated colonic bacteria from sows delivered more SCFAs per g fermented substrate than rapidly fermented starch [45]. In addition, because microbial biomass contains a substantial amount of carbohydrates [46], increased microbial growth may coincide with incorporation of a proportion of polysaccharides into microbial biomass as was shown in rumen bacteria [47]. Consequently, rapid substrate fermentation does not necessarily increases the NE value of a substrate. This was demonstrated by Rijnen*, et al.* [48], who showed that *in vitro* fermentation characteristics of fermentable feed ingredients where unrelated to their *in vivo* NE values. Because microbial produced SCFAs are absorbed and serve as energy source, while microbial protein is lost with feces, bacteria with a low growth efficiency combined with a large SCFA production are preferred in pigs. However, energy losses due to microbial fermentation are small compared to fecal energy losses with unfermented material. Therefore, RS, which is completely fermented in the hindgut of pigs, may still be preferred as energetic feed ingredient above other undigested (by host-enzymes) but not completely fermented carbohydrates.

In general, microbial fermentation deficiency accounts for 10 to 15% of the difference in net energy between fermented and digested carbohydrates. As discussed above, this number

Chapter 7

varies depending on the substrate fermented. For example, RS does not increase methane production, therefore energetic microbial losses with RS intake may be reduced with 4 to 7% when compared to other fermentable carbohydrates.

7.3.3 Energy requirements

It is suggested that high fiber diets increase heat production through additional energy costs required for ingestion, digestion, and metabolism in the gut [49, 50]. The latter is in line with the greater empty gut weight in pigs fed high RS diets (Chapter 2), which is associated with a greater basal heat production [51-53]. It is however, also suggested that fermentable carbohydrates have a positive effect on gut health [54-56], which in turn may decrease the energetic costs for immune system. Particularly RS may be beneficial for maintenance of gut health and reduction of risk factors for intestinal problems [57] through its increasing effect on microbial production of butyrate [58, 59]. In addition, part of the greater heat production in pigs fed fermentable carbohydrates is compensated for by reduced physical activity. Restrictedly-fed pigs fed sugar beet pulp silage [60], raw potato starch [35, 42, 61], or retrograded maize starch [7] were less active than pigs fed digestible starch. Also in Chapter 3 we observed that restrictedly-fed pigs were less active after the afternoon meal with slowly than with rapidly digestible starch. The decrease in activity is suggested to be associated with increased satiety related to gut fill [42] or to a prolonged supply of energy through absorbed SCFAs from microbial fermentation [61]. Because feed intake levels of pigs with *ad libitum* access to feed are higher than with restricted feeding, it can be expected that pigs are more satiated throughout the day regardless of the diet consumed. This may attenuate the sparing effect of fermentable carbohydrates on physical activity. In Chapter 3 however, energy expenditure on physical activity of group-housed pigs that had *ad libitum* access to feed in week 1 did not change when they were restrictedly fed in week 2, which is in line with Collin*, et al.* [62] who observed no effect of feeding level on activity. These results suggest that increased satiety does not inevitably result in less activity; hence, the reducing effect of RS on activity may as well be related to other behavior than foraging behavior [61]. Consequently, the expected increase in basal heat production may also be compensated for by reduced activity under *ad libitum* conditions partly explaining the similar growth rates for low and high RS-fed pigs in Chapter 2.

7.3.4 Heat increment of SCFA versus glucose

The efficiency in which energy from digested nutrients is used for ER is determined by their heat increment, i.e., the energetic costs that are required for nutrient metabolism. The heat increment of fat, carbohydrate, and protein in pigs are approximately 12%, 16%, and 48% of their ME value, respectively [33]. However, the heat increment of each nutrient is a combination of its use for both energy retention and ATP synthesis. Consequently, heat

increments are variable as they are affected by the metabolic fate of absorbed nutrients, which is influenced by, among other factors, maintenance energy requirements, nutrient supply, the physiological state of the pig (fasting versus feeding), and the ratio between body protein and lipid deposition, i.e., growth stage of the pig. To illustrate this, when an animal is housed below its lower critical temperature, incremental ME intake coincides with an increase in ER of the same magnitude, indicating that it is utilized with an efficiency of 100% (heat increment = 0) regardless of the nutrients supplied; all heat that is produced by the animal is used to maintain body temperature. Based on stoichiometric calculations, ATP is less efficiently synthesized (ATP: $\Delta G_{cellular}$ = 50.3 kJ [63]) from absorbed SCFAs (~59% heat increment) than from glucose (68% heat increment; [64]), whereas SCFAs are more efficiently used for fat retention (~10% heat increment; [65]) than glucose (26% heat increment; [64]). Consequently the efficiency in which SCFAs and glucose are used for NE depend on their metabolic fate. When digestible carbohydrates are substituted for indigestible but fermentable carbohydrates, postprandial glucose absorption drops, and less glucose is available for oxidation or *de novo* fatty acid synthesis. Concomitantly, more SCFAs from microbial fermentation will gradually become available for the pig. Both glucose and SCFAs can be used as oxidation substrate throughout the day, but mainly during the fasting period, when less dietary glucose is available to be oxidized, the pig will rely on SCFA oxidation for energy (Chapter 3). This will particularly become apparent in meal-fed pigs, as these pigs are exposed to a longer period of fasting than pigs that have *ad libitum* access to feed. In Chapter 3, we observed that glucose was continuously stored as fat throughout the day in *ad libitum*-fed pigs (RQ>1) indicating that the fasting state may never be reached under these conditions. Although the dietary glucose load of both diets fed in Chapter 3 was probably higher than the high RS diet fed in Chapter 2, the supply of dietary glucose may still have been substantial in high RS fed pigs due to a high inclusion level of dietary starch (50%) and *ad libitum* feeding conditions. Consequently, high RS fed pigs relied less on microbial SCFA production for energy gain under *ad libitum* conditions than when they would have been fed restrictedly, which may have changed the metabolic fate of SCFAs from energy production to fat accretion thereby reducing its heat increment. As a result, the difference between the efficiency in which digestible and fermentable carbohydrates are used for ER may be smaller under *ad libitum* than under restricted conditions. Because currently efficiencies of digested and fermented carbohydrates are only estimated in meal-fed pigs, reported NE values of feed ingredients may be biased by these conditions.

Based on the inefficiency of microbial production (10-15%), the efficiency to metabolize absorbed glucose compared to SCFAs must be 15-20% lower in order to explain the 30% difference in NE between fermented and digested carbohydrates. By additional GE supply via infusion of SCFAs in the caecum, Jørgensen*, et al.* [66] showed that the heat increment

Chapter 7

of SCFAs was 18% of its energy value. Van Milgen, *et al.* [33] showed that the heat increment for starch was 15%. Consequently, the 15-20% difference in NE between digested and fermented carbohydrates unaccounted for by microbial inefficiency is probably not only explained by a difference in heat increment between SCFAs and glucose.

7.4 Alterations in meal patterns

Throughout our studies (Chapter 2 and 5) feeding patterns of growing pigs followed a clear circadian bimodal rhythm when feed was available *ad libitum*, which was consistent with earlier findings [67, 68] and with activity patterns of wild boars [69, 70]. In practice, however, pigs may deviate from this pattern, e.g., due to limited feeder space [71] or high ambient temperatures [72, 73]. In chapter 6, we showed that pigs fed during the night (22.00h and 06.00h) had a 7% greater fat deposition than pigs fed during the day (10.00h and 18.00h). These results indicate that energy utilization of pigs is affected by forced changes in meal pattern. To test the relevance of this concept for common pig practice, InraPorc (INRA, France) was used to simulate growth performance, and more specifically fat deposition, of pigs that eat during the day according to their "normally" feed intake pattern and of pigs that are forced to eat during the night for the entire grower to finish period (30 kg – 110 kg). By reducing the input 'maintenance correction' from 1 to 0.9, a difference in daily fat deposition of ~7% was created without compromising daily protein deposition (Table 7.1). This resulted in 1.7 kg (6%) more fat deposition for pigs that are forced to eat during the night than for pigs that eat during the day. Consequently, back-fat thickness increased with 0.6 mm and lean meat decreased with 1.1 %-units in night-fed pigs while carcass weight was the same for both groups (~92 kg) (Table 7.1). In our study, feed intake was restricted by meals and time, and therefore probably lower than it could have been under *ad libitum* feeding conditions. Consequently, the effect of forced deviations in feeding patterns on fat deposition may even be greater in practice than observed in Chapter 6. Above calculations suggest that changes in meal patterns may impact growth performance of pigs. It is however unclear, if growth performance can be steered by directing meal patterns using feeding strategies. This depends on whether meal patterns are determined by dietary properties or by animal-intrinsic effects. In the next paragraphs, the effects of digestible and resistant starch intake and the influence of animal-intrinsic effects on meal patterns will be discussed.

Table 7.1 Model parameters and results simulated with InraPorc (Inra, France) describing finishing pigs (30 kg – 115 kg) that eat during the day according to their "normal" meal pattern and pigs that are forced to eat night.

	Day feeding	Night feeding
Model parameters		
Initial body weight, kg	30	30
Initial protein mass, kg	4.48	4.48
Initial fat mass, kg	4.38	4.38
Final body weight, kg	115	115
Dressing percentage, %	79	79
Ad libitum intake	$a \cdot BW^b$	$a \cdot BW^b$
a	1.74	1.74
b	0.67	0.67
MeanPD, g/d	164	164
Precocity. 1/d	0.016	0.016
Maintenance correction	1	0.9
Simulated results		
Final body weight, kg	116.6	116.9
Final protein mass, kg	17.1	17.0
Final fat mass, kg	34.0	35.7
Daily performance		
Feed intake, g/d	3019	3021
Growth, g/d	1125	1145
Protein deposition, g/d	164	164
Fat deposition, g/d	386	412
Feed-to-gain ratio, kg feed/kg gain	2.68	2.64
Slaughter characteristics		
Dressing, %	79	79
Carcass weight, kg	92.4	92.1
Back fat thickness, mm	19.7	20.3
Meat, %	53	51.9

7.4.1 Effect of digested and fermented starch on meal patterns

It is suggested in both humans [74, 75] and pigs [76] that absorbed SCFAs from microbial starch fermentation prolong the energy supply to the body postponing the feeling of hunger, whereas a greater insulin release as a response to increased postprandial glucose appearance initiates the ending of a meal. However, despite a substantial difference in ileal starch disappearance between diets, RS intake did not alter meal patterns of growing pigs in the studies of this thesis (Chapter 2 and 5). In addition, in Chapter 5 we concluded that inadequate adaptation to RS intake, rather than RS itself changes meal patterns of pigs. These results are in contrast with Da Silva, *et al.* [76] who reported a greater meal size and

Chapter 7

lower meal frequency in pigs fed retrograded tapioca (high-RS) than pigs fed pre-gelatinized potato starch (low-RS). In Chapter 2 we discussed that the difference in results between our study and the study of Da Silva, *et al.* [76] may be related to the difference in starch digestibility between the RS sources used. The greater extent of starch digestion of the high amylose maize starch (73%) than tapioca (54%) resulted probably in a higher glucose but lower SCFA supply. Consequently, high amylose maize starch and tapioca may affect satiety regulating mechanisms differently (Chapter 1). In pigs that were fed a diet containing high amylose starch [6], postprandial release of satiety regulating hormones GLP-1 and insulin decreased, while SCFA levels increased [77]. The lack of response in meal patterns to dietary RS intake in Chapter 2 and 5, may suggest that these satiety-controlling mechanisms are less important for feed intake regulation of pigs than previously assumed.

7.4.2 Animal-intrinsic effects on meal patterns

Feeding behavior of pigs is affected by intrinsic factors such as age [78, 79], gender (Chapter 2, and [80]), and genotype [81-83], but also by environmental factors such as group size [80], ambient temperature [72], and behavioral cues, such as the light/dark schedule [84]. In this thesis, meal patterns of individual pigs were recorded for 4 months (Chapter 2 and 5 combined), in which pigs were housed under controlled environmental conditions (artificial lighting, set temperature, fixed group size). Pigs were fed different dietary treatments during this period, however, RS did not affect meal patterns of pigs in Chapter 2 and reported effects in Chapter 5 were rather small. Because environmental effects on meal patterns were most likely minor during the entire period, I was able to investigate the animal-intrinsic effect on the pig's meal pattern by analyzing the consistency in meal frequency of individual pigs over time. Meal frequency was selected for this analysis, because it was not affected by any of the dietary treatments used in both studies (Chapter 2 and 5). Based on its meal frequency at a body weight (BW) of 30 kg, a pig was defined as meal eater (low number of meals), average, or nibbler (high number of meals). Subsequently, the percentage of pigs that switched from their initial assigned category at BW 30 kg was analyzed at a BW of 65 and 110 kg (Figure 7.4). Because a switch to or from the average meal frequency category may result from a minor change in meal frequency, particularly switches between the most extreme categories (meal eaters and nibblers) are of interest. Approximately 15% and 20% of the pigs that were initially defined as a meal eater or a nibbler switched to the opposite (extreme) category at a BW of 65 and 110 kg, respectively (Figure 7.4). Of the initial defined meal eaters and nibblers, approximately 60% at a BW of 65 kg and 50% at a BW of 110 kg were still defined as a meal eater or nibbler. These results show that meal patterns of most pigs stay consistent over time, and that only a low number of pigs (~10% of the total pig population) change their meal pattern drastically, which may suggest that meal patterns of pigs are partly explained by animal-intrinsic effects. In chapter 3, we observed that reduced

feed intake rather than reduced feed efficiency explains slow growth of low performance pigs. Understanding individual variation in feeding behaviour may help us to further explain variation in growth performance that exists among pigs in practice.
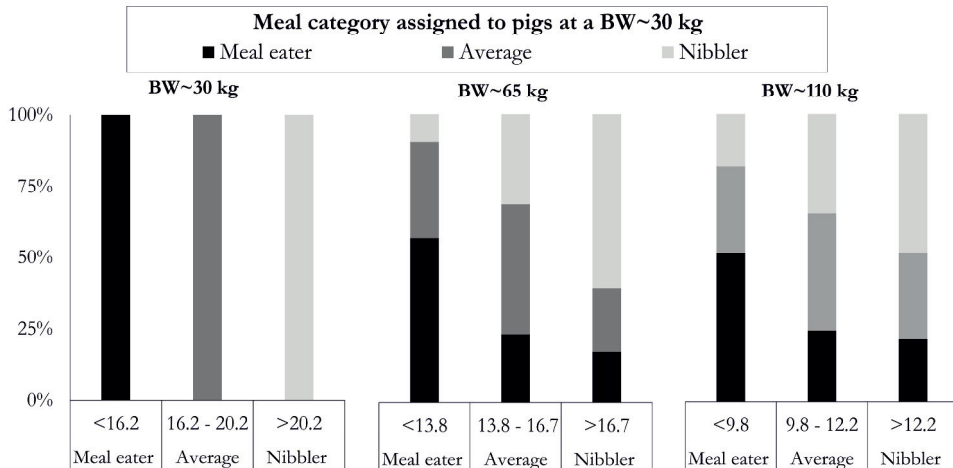

Figure 7.4 Development of meal frequency of growing pigs (BW 30-110 kg). At a BW of 30, 65, and 110 kg, the population was equally divided in three meal categories based on the number of meals; meal eater, average eater, and nibbler. The meal category assigned to pigs at a BW of 30 kg was used as a reference (initial meal pattern); meal eaters (black), average (grey) or nibblers (light grey). Subsequently, the percentage of pigs of these initial categories observed to be meal eaters, average or nibblers at 65 kg of BW and 110 kg of BW was calculated.

7.5 Main conclusions

The main conclusions to be drawn from this thesis are:

- Rapidly digestible starch favors postprandial lipogenesis ultimately leading to an increase in fat deposition in growing pigs.
- The extent of starch digestion, hence resistant starch (RS) intake does not impact growth performance in *ad libitum* fed pigs indicating that net energy values of RS and digestible starch are similar.
- Slow growth in healthy normal-birth-weight pigs is explained by reduced feed intake rather than reduced feed efficiency.
- Between 2 and 7 % of the starch consumed is fermented in the upper gastrointestinal tract of growing pigs.
- Inadequate adaptation to high dietary RS concentrations decreases voluntary feed intake of growing pigs.

Chapter 7

- Meal patterns of growing pigs (once adapted) are not affected by substantial changes in RS intake (30% in diet).
- Intestinal microbiota adapts quickly to increases in RS intake but keeps fermenting starch when RS intake decreases, consequently fermenting digestible starch.
- Night feeding impacts energy utilization in growing pigs ultimately increasing fat deposition.
- Night feeding increases methane production in growing pigs.

7.6 Implications

Starch digestion kinetics

This thesis describes the impact of starch digestion kinetics on pig performance. We showed that the difference in the extent of starch digestion, and thus dietary RS inclusion, did not affect growth rate, back-fat thickness, and leanness, whereas a slower rate of starch digestion decreased fat deposition of pigs. The latter may be used as a feeding application to decrease fat deposition in growing pigs, which negatively affects the pig farmer's income. However, results in this thesis are obtained by feeding a starch source that may have a slower rate of starch digestion than starch sources commonly used for pig feed. Therefore, to investigate the applicability of the rate of starch digestion in feed formulation, its variation among feed ingredients used in pig feed, and, subsequently, the magnitude of its effect on pig performance within this variation needs to be assessed. When doing so, the rate of starch digestion (g starch digested/min) should be expressed as a proportion of total starch digested to allow for comparison between feed ingredients, because slowly digestible starch sources often contain higher contents of RS than rapidly digestible starch sources. Furthermore, slowly digestible starch may be relevant for pigs exposed to high ambient temperatures. A slower rate of starch digestion decreases postprandial heat production (Chapter 3). This application may particularly be of interest in pigs exposed to heat stress that are fed a low number of meals per day.

Starch fermentation

Research presented in this thesis showed that the contribution of starch fermentation to total starch disappearance is substantial indicating that currently ileal starch digestion is overestimated. Future research should focus on microbial activity and starch fermentation in the upper gastrointestinal tract and its contribution to small intestinal health and overall energy supply for the pig. The latter, however, may be less relevant for specific conditions as this thesis showed that under *ad libitum* conditions digested and fermented starch appear to deliver the same amount of energy for growth. Results in chapter 5 showed that pigs ferment part of the potentially digestible starch when switching from a high RS diet to a low

RS diet. These results may be relevant for diet transitions in practice, e.g., when sows switch from a gestation feed to a lactation feed. Diets for gestating sows typically contain higher concentrations of fermentable ingredients than diets for lactating sows, which stimulates the sow's fermentative capacity affecting the utilization of the subsequent diet.

Improvement of current energy systems

In this thesis it was shown that the NE value of RS and digestible starch are more similar than commonly assumed, specifically in group-housed pigs fed under *ad libitum* conditions. These results indicate that NE predictions are affected by housing and feeding conditions. In addition, due to existing interactions between nutrients and feed ingredients NE values of feed ingredients may not be additive when used in a feed matrix. In order to improve future energy predictions, feed evaluation systems should account for varying conditions related to the pig (i.e., age, sex, growth stage), environment (i.e., feeding system, ambient temperature), and the feed ingredient and diet consumed (i.e., interaction between nutrients and feed ingredients) that exist in practice. Some of these variables may be accounted for by adjustments in energy requirements, e.g. animal-related and some environmental effects, other, diet related effects, should be accounted for by energy predictions. Because it is quite a challenge to include these variables in current energy predictions, a feed ingredient evaluation system based on DE rather than NE may be more suitable for currently common pig practice. The DE value of ingredients captures most of the variation that exists between feed ingredients, and is probably less affected by environmental conditions than NE. It should be noted, though, that existing DE values of ingredients containing indigestible carbohydrates may be underestimated as well due to incorrect estimates of digested and fermented energy related to the shift from urinary to fecal energy losses.

Time of feeding

Daily feed intake is an important indicator of pig performance and health, but results of this thesis showed the timing of feed intake to be important for pigs as well (Chapter 6). Forcing pigs to feed during the night negatively affects growth performance by increasing fat deposition, but might as well have negative consequences for health as was shown in humans and rodents. Despite the extensive number of human and rodent studies executed, still major gaps in the understanding of circadian misalignment exist, which should further be investigated. Timing of feed intake in relation to performance is particularly of interest for pigs exposed to a high stocking density or high ambient temperatures, but may certainly also be relevant for, e.g., gestating sows that in practice often are fed only once or twice a day. Also pigs fed via liquid feeding systems often receive only a few meals per day in a matter convenient for the farmer, not for the biology of the pig. For these systems, it might be of interest to minimize feed supply during the night.

Chapter 7

Feeding strategies to influence meal patterns of pigs

Results of this thesis showed that replacing digestible starch with RS (30% dietary RS inclusion) does not change meal patterns of growing pigs. Consequently, RS is not suitable as a feed application to steer feeding behavior of pigs, particularly when considering that common pig diets contain lower RS concentrations than the diets used in this thesis. In addition, meal patterns of pigs might partly be affected by animal-intrinsic effects. When the pig's meal pattern is truly an animal-intrinsic property, feeding solutions may still be relevant for improving growth performance in genetic lines that are characterized by specific feeding behavior.

References

1. Van Kempen T, Pujol S, Tibble S, Balfagon A: *In vitro* characterization of starch digestion and its implications for pigs. In *Paradigms in Pig Science*. Edited by Wiseman J, Varley M, Mcorist S, Kemp B. Nottingham: Nottingham University Press; 2007: 515-525

2. Fouhse JM, Zijlstra RT: Impact of resistant vs. digested starch on starch energy value in the pig gut. *Bioact Carbohydr Dietary Fibre* 2018, 15:12-20.

3. Martens BM, Flécher T, De Vries S, Schols HA, Bruininx EM, Gerrits WJ: Starch digestion kinetics and mechanisms of hydrolysing enzymes in growing pigs fed processed and native cereal based diets. *Br J Nutr* 2019:1-29.

4. Thomas M, Huijnen PTHJ, Van Vliet T, Van Zuilichem DJ, Van der Poel AFB: Effects of process conditions during expander processing and pelleting on starch modification and pellet quality of tapioca. *J Sci Food Agric* 1999, 79:1481-1494.

5. Holm J, Lundquist I, Björck I, Eliasson A-C, Asp N-G: Degree of starch gelatinization, digestion rate of starch *in vitro*, and metabolic response in rats. *Am J Clin Nutr* 1988, 47:1010-1016.

6. Van Kempen TA, Regmi PR, Matte JJ, Zijlstra RT: *In vitro* starch digestion kinetics, corrected for estimated gastric emptying, predict portal glucose appearance in pigs. *J Nutr* 2010, 140:1227-1233.

7. Gerrits WJ, Bosch MW, Van den Borne JJ: Quantifying resistant starch using novel, *in vivo* methodology and the energetic utilization of fermented starch in pigs. *J Nutr* 2012, 142:238-244.

8. Fouhse JM, Gänzle MG, Regmi PR, Van Kempen TA, Zijlstra RT: High amylose starch with low *in vitro* digestibility stimulates hindgut fermentation and has a bifidogenic effect in weaned pigs. *J Nutr* 2015, 145:2464-2470.

9. Bohn T, Carriere F, Day L, Deglaire A, Egger L, Freitas D, Golding M, Le Feunteun S, Macierzanka A, Menard O: Correlation between *in vitro* and *in vivo* data on food digestion. What can we predict with static *in vitro* digestion models? *Crit Rev Food Sci Nutr* 2018, 58:2239-2261.

10. Hasjim J, Lavau GC, Gidley MJ, Gilbert RG: *In vivo* and *in vitro* starch digestion: are current *in vitro* techniques adequate? *Biomacromolecules* 2010, 11:3600-3608.

11. Martens BM, Bruininx E, Gerrits WJJ, Schols H: The importance of gastric amylases in starch digestion kinetics in pigs. In *Starch digestion kinetics in pigs: The impact of starch stucture, feed processning, and digesta passage behaviour*. Edited by Martens BM. Wageningen2019

12. Van der Meulen J, Bakker G, Bakker J, De Visser H, Jongbloed A, Everts H: Effect of resistant starch on net portal-drained viscera flux of glucose, volatile fatty acids, urea, and ammonia in growing pigs. *J Anim Sci* 1997, 75:2697-2704.

13. Deutz N, Ten Have G, Soeters P, Moughan P: Increased intestinal amino-acid retention from the addition of carbohydrates to a meal. *Clin Nutr* 1995, 14:354-364.

14. Vangsøe CT, Ingerslev AK, Theil PK, Hedemann MS, Lærke HN, Knudsen KEB: *In vitro* starch digestion kinetics of diets varying in resistant starch and arabinoxylan compared with *in vivo* portal appearance of glucose in pigs. *Food Research International* 2016, 88:199-206.

15. Noah L, Krempf M, Lecannu G, Maugère P, Champ M: Bioavailability of starch and postprandial changes in splanchnic glucose metabolism in pigs. *Am J Physiol Endocrinol Metab* 2000, 278:E181-E188.

16. Doti S, Suárez-Belloch J, Latorre M, Guada J, Fondevila M: Effect of dietary starch source on growth performances, digestibility and quality traits of growing pigs. *Livest Sci* 2014, 164:119-127.

Chapter 7

17. Li T, Huang R, Wu G, Lin Y, Jiang Z, Kong X, Chu W, Zhang Y, Kang P, Hou Z: Growth performance and nitrogen metabolism in weaned pigs fed diets containing different sources of starch. *Livest Sci* 2007, 109:73-76.

18. Bolhuis J, Van den Brand H, Staals S, Gerrits W: Effects of pregelatinized vs. native potato starch on intestinal weight and stomach lesions of pigs housed in barren pens or on straw bedding. *Livest Sci* 2007, 109:108-110.

19. Rijnen M, Dekker R, Bakker G, Verstegen M, Schrama J: Effects of dietary fermentable carbohydrates on the empty weights of the gastrointestinal tract in growing pigs. *Digestive Physiology of Pigs Eds: Lindberg, JE & Ogle, B, CABI Publishing, New York* 2001:17-20.

20. Jensen BB: The impact of feed additives on the microbial ecology of the gut in young pigs. *J Anim Feed Sci* 1998, 7:45-64.

21. Kidder D, Manners M: The level and distribution of carbohydrases in the small intestine mucosa of pigs from 3 weeks of age to maturity. *Br J Nutr* 1980, 43:141-153.

22. Owsley W, Orr Jr D, Tribble L: Effects of age and diet on the development of the pancreas and the synthesis and secretion of pancreatic enzymes in the young pig. *J Anim Sci* 1986, 63:497-504.

23. Lindemann M, Cornelius S, El Kandelgy S, Moser R, Pettigrew J: Effect of age, weaning and diet on digestive enzyme levels in the piglet. *J Anim Sci* 1986, 62:1298-1307.

24. Jensen MS, Jensen SK, Jakobsen K: Development of digestive enzymes in pigs with emphasis on lipolytic activity in the stomach and pancreas. *J Anim Sci* 1997, 75:437-445.

25. Blok MC, Brandsma G, Bosch G, Gerrits WJJ, Jansman AJM, Fledderus J, Everts H: A new dutch net energy formula for feed and feedstuffs for growing and fattening pigs. In *CVB-Documentation Report*. Wageningen, the Netherlands: Centraal Veevoeder Bureau; 2015.

26. NRC: *Nutrient requirements of swine.* Washington, D.C., USA: National Academic Press; 2012.

27. Blaxter K: *Energy metabolism in animals and man.* Cambridge (United Kingdom): Cambridge University Press; 1989.

28. Just A, Fernández J, Jørgensen H: The net energy value of diets for growth in pigs in relation to the fermentative processes in the digestive tract and the site of absorption of the nutrients. *Livest Prod Sci* 1983, 10:171-186.

29. Jørgensen H, Zhao X-Q, Eggum BO: The influence of dietary fibre and environmental temeporature on the development of the gastrointestinal tract, digestibility, degree of fermentation in the hind-gut and energy metabolism in pigs. *Br J Nutr* 1996, 75:365-378.

30. Noblet J, Fortune H, Shi XS, Dubois S: Prediction of net energy value of feeds for growing pigs. *J Anim Sci* 1994, 72:344-354.

31. Wilfart A, Montagne L, Simmins P, Van Milgen J, Noblet J: Sites of nutrient digestion in growing pigs: Effect of dietary fiber 1. *J Anim Sci* 2007, 85:976-983.

32. de Vries S, Gerrits WJJ, Kabel MA, Vasanthan T, Zijlstra RT: β-Glucans and resistant starch alter the fermentation of recalcitrant fibers in growing pigs. *PLoS One* 2016, 11:e0167624.

33. Van Milgen J, Noblet J, Dubois S: Energetic efficiency of starch, protein and lipid utilization in growing pigs. *J Nutr* 2001, 131:1309-1318.

34. Heijnen M-LA, Beynen AC: Consumption of retrograded (RS3) but not uncooked (RS2) resistant starch shifts nitrogen excretion from urine to feces in cannulated piglets. *J Nutr* 1997, 127:1828-1832.

35. Bolhuis JE, Van den Brand H, Staals STM, Zandstra T, Alferink SJJ, Heetkamp MJW, Gerrits WJJ: Effects of fermentable starch and straw-enriched housing on energy partitioning of growing pigs. *Animal* 2008, 2:1028-1036.

36. Mosenthin R, Sauer W, Henkel H, Ahrens F, De Lange C: Tracer studies of urea kinetics in growing pigs: II. The effect of starch infusion at the distal ileum on urea recycling and bacterial nitrogen excretion. *J Anim Sci* 1992, 70:3467-3472.

37. Rumble J: *CRC handbook of chemistry and physics*. CRC press; 2017.

38. Hungate RE: Quantities of carbohydrate fermentation products. In *The rumen and its microbes*. London: Academic Press Inc.; 1966

39. Noblet J, Jondreville C: Valeurs nutritives pour les porcs. In *Tables de composition et de valeur nutritive des matières premières destinées aux animaux d'élevage: porcs, volailles, bovins, ovins, caprins, lapins, chevaux, poissons*. Edited by Sauvant D, Perez J, Tran G. Versailles: INRA Editions; 2002

40. Rijnen M, Verstegen M, Schrama J: Effects of two different sources of dietary fiber on energy metabolism in group-housed growing pigs. In *Energetic utilization of dietary fiber in pigs*. Edited by Rijnen M. Wageningen2003

41. Jensen BB, Jørgensen H: Effect of dietary fiber on microbial activity and microbial gas production in various regions of the gastrointestinal tract of pigs. *Appl Environ Microbiol* 1994, 60:1897-1904.

42. Schrama JW, Bakker GC: Changes in energy metabolism in relation to physical activity due to fermentable carbohydrates in group-housed growing pigs. *J Anim Sci* 1999, 77:3274-3280.

43. Gibson G, Cummings J, Macfarlane G, Allison C, Segal I, Vorster H, Walker A: Alternative pathways for hydrogen disposal during fermentation in the human colon. *Gut* 1990, 31:679-683.

44. Govers MJAP, Gannon NJ, Dunshea FR, Gibson PR, Muir JG: Wheat bran affects the site of fermentation of resistant starch and luminal indexes related to colon cancer risk: a study in pigs. *Gut* 1999, 45:840-847.

45. Bindelle J, Buldgen A, Wavreille J, Agneessens R, Destain J-P, Wathelet B, Leterme P: The source of fermentable carbohydrates influences the *in vitro* protein synthesis by colonic bacteria isolated from pigs. *Animal* 2007, 1:1126-1133.

46. Storm E, Ørskov ER: The nutritive value of rumen micro-organisms in ruminants: 1. Large-scale isolation and chemical composition of rumen micro-organisms. *Br J Nutr* 1983, 50:463-470.

47. Hungate R: Polysaccharide storage and growth efficiency in Ruminococcus albus. *J Bacteriol* 1963, 86:848-854.

48. Rijnen MMJA, Schrama JW, Van den Borne JJGC, Gerrits WJJ, Cone JW, Van Gelder A: Effecten van fermenteerbare grondstoffen op de fysieke activiteit in relatie tot energiebenutting bij varkens. Wageningen: Wageningen University; 2004.

49. Le Goff G, Van Milgen J, Noblet J: Influence of dietary fibre on digestive utilization and rate of passage in growing pigs, finishing pigs and adult sows. *Anim Sci* 2002, 74:503-515.

50. Varel V, Yen JT: Microbial perspective on fiber utilization by swine. *J Anim Sci* 1997, 75:2715-2722.

51. Koong L, Ferrell C, Nienaber J: Assessment of interrelationships among levels of intake and production, organ size and fasting heat production in growing animals. *J Nutr* 1985, 115:1383-1390.

52. de Lange K, Van Milgen J, Noblet J, Dubois S, Birkett S: Previous feeding level influences plateau heat production following a 24 h fast in growing pigs. *Br J Nutr* 2006, 95:1082-1087.

53. Labussière E, Van Milgen J, de Lange CF, Noblet J: Maintenance energy requirements of growing pigs and calves are influenced by feeding level. *J Nutr* 2011, 141:1855-1861.

54. Lindberg JE: Fiber effects in nutrition and gut health in pigs. *J Anim Sci Biotechno* 2014, 5:15.

Chapter 7

55. Bindelle J, Leterme P, Buldgen A: Nutritional and environmental consequences of dietary fibre in pig nutrition: a review. *Biotechnologie, Agronomie, Société et Environnement* 2008, 12:69-80.

56. Jha R, Berrocoso J: Dietary fiber utilization and its effects on physiological functions and gut health of swine. *Animal* 2015, 9:1441-1452.

57. Brouns F, Kettlitz B, Arrigoni E: Resistant starch and "the butyrate revolution". *Trends Food Sci Technol* 2002, 13:251-261.

58. Mentschel J, Claus R: Increased butyrate formation in the pig colon by feeding raw potato starch leads to a reduction of colonocyte apoptosis and a shift to the stem cell compartment. *Metabolism* 2003, 52:1400-1405.

59. Martin LJ, Dumon HJ, Lecannu G, Champ MM: Potato and high-amylose maize starches are not equivalent producers of butyrate for the colonic mucosa. *Br J Nutr* 2000, 84:689-696.

60. Schrama J, Verstegen M, Verboeket P, Schutte J, Haaksma J: Energy metabolism in relation to physical activity in growing pigs as affected by type of dietary carbohydrate. *J Anim Sci* 1996, 74:2220-2225.

61. Bolhuis JE, Van den Brand H, Bartels AC, Oostindjer M, Van den Borne JJ, Kemp B, Gerrits WJ: Effects of fermentable starch on behaviour of growing pigs in barren or enriched housing. *Appl Anim Behav Sci* 2010, 123:77-86.

62. Collin A, Van Milgen J, Dubois S, Noblet J: Effect of high temperature and feeding level on energy utilization in piglets. *J Anim Sci* 2001, 79:1849-1857.

63. Cooper M: *The Cell: a molecular approach.* American society of microbiology; 2000.

64. Black J: Modelling energy metabolism in the pig-critical evaluation of a simple reference model. 1995.

65. Hungate RE: Host metabolism and rumen processes. In *The rumen and its microbes.* London: Academic Press Inc.; 1966

66. Jørgensen H, Larsen T, Zhao X-Q, Eggum BO: The energy value of short-chain fatty acids infused into the caecum of pigs. *Br J Nutr* 1997, 77:745-756.

67. De Haer L, Merks J: Patterns of daily food intake in growing pigs. *Anim Sci* 1992, 54:95-104.

68. Bornett H, Morgan C, Lawrence A, Mann J: The effect of group housing on feeding patterns and social behaviour of previously individually housed growing pigs. *Appl Anim Behav Sci* 2000, 70:127-141.

69. Gundlach H: Verhaltensontogenese und tagesperiodik beim europaishen wildaischwein (Sus scrofa L). *Zeitschrift Tierpsychologie* 1968, 25:955-995.

70. D'Eath RB, Turner SP: The natural behaviour of the pig. In *The welfare of pigs.* Springer; 2009: 13-45

71. Nielsen BL, Lawrence AB, Whittemore CT: Effect of group size on feeding behaviour, social behaviour, and performance of growing pigs using single-space feeders. *Livest Prod Sci* 1995, 44:73-85.

72. Quiniou N, Dubois S, Noblet J: Voluntary feed intake and feeding behaviour of group-housed growing pigs are affected by ambient temperature and body weight. *Livest Prod Sci* 2000, 63:245-253.

73. Feddes J, Young B, DeShazer J: Influence of temperature and light on feeding behaviour of pigs. *Appl Anim Behav Sci* 1989, 23:215-222.

74. Anderson GH, Catherine NL, Woodend DM, Wolever TM: Inverse association between the effect of carbohydrates on blood glucose and subsequent short-term food intake in young men. *Am J Clin Nutr* 2002, 76:1023-1030.

75. Nilsson AC, Östman EM, Holst JJ, Björck IM: Including indigestible carbohydrates in the evening meal of healthy subjects improves glucose tolerance, lowers inflammatory markers, and increases satiety after a subsequent standardized breakfast. *J Nutr* 2008, 138:732-739.

76. Da Silva CS, Bosch G, Bolhuis J, Stappers L, Van Hees H, Gerrits W, Kemp B: Effects of alginate and resistant starch on feeding patterns, behaviour and performance in *ad libitum*-fed growing pigs. *Animal* 2014, 8:1917-1927.

77. Regmi PR, Metzler-Zebeli BU, Gänzle MG, Van Kempen TA, Zijlstra RT: Starch with high amylose content and low *in vitro* digestibility increases intestinal nutrient flow and microbial fermentation and selectively promotes bifidobacteria in pigs. *J Nutr* 2011, 141:1273-1280.

78. Nienaber J, McDonald T, Hahn G, Chen Y: Eating dynamics of growing-finishing swine. *Transactions of the ASAE* 1990, 33:1211-1218.

79. Hyun Y, Ellis M, McKeith F, Wilson E: Feed intake pattern of group-housed growing-finishing pigs monitored using a computerized feed intake recording system. *J Anim Sci* 1997, 75:1443-1451.

80. Hyun Y, Ellis M: Effect of group size and feeder type on growth performance and feeding patterns in finishing pigs. *J Anim Sci* 2002, 80:568-574.

81. De Haer L, De Vries A: Effects of genotype and sex on the feed intake pattern of group housed growing pigs. *Livest Prod Sci* 1993, 36:223-232.

82. Labroue F, Guéblez R, Meunier-Salaün M-C, Sellier P: Feed intake behaviour of group-housed Piétrain and Large White growing pigs. In *Annales de zootechnie* 1999: 247-261.

83. Fàbrega E, Tibau J, Soler J, Fernández J, Font J, Carrión D, Diestre A, Manteca X: Feeding patterns, growth performance and carcass traits in group-housed growing-finishing pigs: the effect of terminal sire line, halothane genotype and age. *Anim Sci* 2003, 77:11-21.

84. Boumans IJ, de Boer IJ, Hofstede GJ, la Fleur SE, Bokkers EA: The importance of hormonal circadian rhythms in daily feeding patterns: An illustration with simulated pigs. *Horm Behav* 2017, 93:82-93.

Chapter 7

Summary

In pigs, starch is digested into glucose in the small intestine. Undigested starch, i.e. resistant starch (RS), serves as substrate for microbial fermentation. The aim of this thesis was to improve understanding of underlying processes affecting the utilization of digested and fermented starch to ultimately predict the effect of starch digestion kinetics on pig performance more accurately.

Despite the assumption that fermented starch yields less energy than digested starch, growth rates of pigs fed low RS and high RS diets are often the same when feed is available *ad libitum*. In **Chapter 2**, we hypothesized that RS affects nutrient digestion and digesta passage rate, and consequently feeding behavior, which ultimately affects growth performance of pigs under *ad libitum* conditions. In this study, pigs were fed either a high RS (HRS) or low RS (LRS) diet. Despite a substantial reduction in enzymatic digestible starch (LRS: 98% vs. HRS: 74%), carcass gain, slaughter quality parameters, and feed efficiency used for carcass gain were not affected by diet. Because dietary RS concentration did not affect digesta passage rate nor feeding behavior, we suggested that the difference in energy intake between fermentable and digestible starch was compensated for post-absorptively.

The aim of **Chapter 3** was to investigate maintenance energy requirements and the efficiency in which energy is used for growth (incremental energy efficiency) in slow- (SG) and fast growing (FG) pigs, which were fed either a slowly (SDS) or a rapidly digestible starch (RDS) diet. A slower rate of starch digestion was hypothesized to reduce fat depositing in pigs and be beneficial for slow growing pigs that are associated with a reduced insulin sensitivity. Gross energy intake was 6% greater in FG-pigs than in SG-pigs, whereas incremental energy efficiencies and fasting heat production were unaffected. We concluded that a lower energy intake rather than greater maintenance requirements or a lower energy efficiency explains slow growth of SG-pigs. No beneficial effects of SDS on energy utilization of SG-pigs were observed. RDS increased incremental use of energy for fat retention (2 %-units), which was most likely explained by greater levels of postprandial fat deposition.

Microbial activity in the small intestine has shown to exist, which may result in an overestimation of starch digestion when not accounted for. In **Chapter 4**, we aimed to quantify both ileal and total tract starch fermentation and investigated the effect of RS on bacterial biomass formation and microbiota composition. A method based on the contrast in natural ^{13}C-enrichment between starch and non-starch dietary components was used to quantify total tract fermentation of starch. Pigs were fed either a high RS (HRS) or low RS (LRS) diet. Microbiota composition in rectal digesta, but not in ileal digesta, slightly differed between diets. Total tract starch fermentation was 18 %-units greater in HRS-fed pigs than LRS-fed pigs ($P<0.001$). Large intestinal starch disappearance was 24 %-units greater in LRS-fed than HRS-fed pigs ($P<0.001$), implying that ileal starch fermentation was 6 %-units greater in HRS-fed pigs than LRS-fed pigs ($P=0.046$). The ^{13}C-method used to estimate

starch fermentation was reasonably close to colonic starch disappearance, but largely overestimated ileal starch fermentation. Our results suggested that ileal starch digestion in pigs can be overestimated with 1-7% when based on ileal starch disappearance.

In **Chapter 5**, we hypothesized that alterations in feeding behavior to changes in RS intake may be dynamic, depending on the adaptation of processes involved when shifting from starch digestion to fermentation or vice versa. To test this hypothesis, we interchanged HRS and LRS in 5 steps, either in upwards (low to high; LH) or downwards (high to low; HL) direction. Complete substitution of LRS with HRS increased the proportion of starch fermented, which was greater in LH pigs (17.6%) than HL pigs (8.18%) and decreased feed intake (106 g/d) and meal size (12.6 g) of LH pigs, but not of HL pigs. We concluded that pigs adapt more slowly to increasing dietary supply of digestible starch than to RS, which resulted in fermentation of potentially enzymatically digestible starch. Furthermore, feed intake decreased only in pigs poorly adapting to RS; hence, the adequacy of adaptation, rather than RS itself reduced feed intake of pigs.

Misalignment of the day/night rhythm with circadian feeding rhythms (circadian misalignment) has been shown to increase fat deposition and the risk for metabolic disorders in humans and rodents. In **Chapter 6**, we investigated the effects of circadian misalignment on energy expenditure in pigs. Pigs were fed either during the day (10.00h - 18.00h; diurnal feeding: DF) or night (22.00h - 06.00h; nocturnal feeding: NF), bihourly the same sequential meals, representing 15, 10, 25, 30 and 20 % of a similar daily allowance. Heat production was 3% lower for NF-pigs than DF-pigs increasing fat retention by 7% in NF-pigs. Methane production was 30% greater in NF-pigs than in DF-pig. We concluded that circadian misalignment has little effect on nutrient digestion, but alters nutrient partitioning, ultimately increasing fat deposition. The causality of the association between circadian misalignment and methane production rates remained to be investigated.

In **Chapter 7**, I discussed the impact of starch digestion kinetics on growth performance, energy utilization, and meal patterns of pigs, by combining the results described in this thesis with existing literature. Rapidly digestible starch favors postprandial lipogenesis ultimately leading to an increase in fat deposition in pigs, whereas the extent of starch digestion, hence RS, did not affect growth performance of pigs. Consequently, net energy values of RS and digestible starch seem to be similar, particularly under *ad libitum* conditions. Finally, meal patterns may be determined by both dietary and animal-intrinsic effects. Substantial changes in RS intake (30% RS), however, did not affect meal patters of growing pigs.

Acknowledgements

Na 4 jaar promotieonderzoek is het einde in zicht, het enige dat me nog rest is het dankwoord schrijven. Ik ben ontzettend trots op hetgeen wat ik uiteindelijk heb opgeleverd. Dit was me nooit gelukt zonder de steun en hulp van alle mensen om me heen.

Allereerst wil ik mijn (co) promotoren bedanken. **Leo,** bedankt voor de mogelijkheid die je me hebt gegeven om een PhD binnen Trouw Nutrition te mogen starten. Daarnaast ook bedankt voor de vrijheid die ik kreeg om mezelf te ontwikkelen en voor al je praktische kennis wat mij erg geholpen heeft om alle resultaten in perspectief te plaatsen. **Walter,** ik ben je nog steeds dankbaar dat je 5 jaar geleden mijn naam hebt laten vallen bij Nutreco waar uiteindelijk mijn PhD project uit is opgestart. Ik kon altijd bij je terecht voor advies of gewoon voor een gezellig praatje, bedankt daar voor. Samen hebben we heel wat mooie plaatjes van grafieken zitten bekijken de afgelopen jaren, en ik weet het, soms wat (te) veel. Bedankt voor al het geduld dat je met me hebt gehad en voor de stimulerende omgeving die je al die tijd hebt gecreëerd om het beste uit mezelf te halen! **Sonja,** ook jou wil ik bedanken voor al je tijd en goede advies gedurende de laatste jaren. Jij en Walter waren de perfecte combi voor een constructieve discussie op de vrijdagmorgen. Ook als het even tegen zat kon ik bij je binnen lopen voor een duwtje in de goede richting. Het is een eer om je eerste PhD student te zijn, en ik weet zeker dat er nog veel zullen volgen! Bedankt! **Theo,** binnen Trouw Nutrition stond je bekend als de zetmeel-expert. Vanzelfsprekend werd je betrokken bij het project vanaf het eerste moment, waarna je na enige tijd mijn manager werd. Ik wil je bedanken voor al jouw tijd en advies, en zeker ook voor de mentor rol die je op je genomen hebt wat er voor heeft gezorgd dat ik binnen Trouw Nutrition mijn PhD heb kunnen voltooien. Bedankt voor je enthousiasme, je creativiteit, en je out-of-the-box mentaliteit wat altijd weer goede input leverde voor discussie.

Naast mijn directe begeleiding heb ik veel hulp gekregen van collega's, zowel van Trouw Nutrition als van ANU. Ook deze mensen zou ik graag willen bedanken voor alle hulp die zij geboden hebben.

Fellow (ex)swine researchers, **Jan Willem**, **Pieter**, **Tetske**, **Neil**, **and Nestor**, thanks for all the discussions that we had about my study designs and results, and for all the advice you have given me the last four years. **Hubert**, bedankt voor het mede mogelijk maken van mijn PhD. Na ongeveer een jaar heb je het stokje overgedragen aan Theo, maar ik waardeer je input en steun gedurende de eerste jaren enorm. **Anke**, **Marleen**, **Kelly**, en **Carlijn**, bedankt voor alle praktische steun tijdens mijn proeven. Zonder jullie was er geen voer geformuleerd, waren er waarschijnlijk geen proeven verricht, en waren zeker niet alle potjes gelabeld. Ook wil ik iedereen bedanken op Halfweg en Carus die mee gewerkt hebben aan mijn proeven. Zonder jullie was het niet gelukt om alle proeven tot een goed einde te brengen! Iedereen van het **ANU lab**, bedankt voor het verwerken en analyseren van alle monsters die zijn

Soms zijn de mensen die het meest ver af staan van waar je elke dag mee bezig bent het meest belangrijk om je even uit het PhD bestaan te trekken. De ontspanning en gezelligheid met vrienden en familie heeft mij met regelmaat mijn PhD stress doen vergeten.

Roel, Melanie, John, Danique, Lieke, Joey, Amy en **Niels**, bedankt voor jullie getoonde interesse en gezelligheid de laatste jaren. Even terug in Brabant was altijd weer een fijne afleiding van mijn werkende leventje in Wageningen. **Roel** en **Melanie**, ook bedankt voor alle spontane avondjes waarbij ik vervolgens kon blijven slapen nadat het na een paar biertjes en goede gesprekken te laat was geworden om naar huis te rijden. **Centaurians**, I would like to thank you all for all the years that we have spend together, and for the great time I had during this period! Without WHV Centauri, Wageningen would have been a completely different experience for me. In het bijzonder wil ik **Simon**, **Nadieh**, en **Steven** bedanken. Wat hebben wij het nog lang vol gehouden bij de club. Bedankt voor alle etentjes, feestjes, en natuurlijk bbqs die we samen hebben gehad! **Roy**, oud centauri-genoot, maat, we hebben de laatste jaren met regelmaat in de bioscoop gezeten, en zo ongeveer alle Marvel films wel gezien. Ik kon altijd bij je terecht voor een avondje gezelligheid, bedankt hiervoor! **Ron**, vakantiemaat, wat zouden de laatste 4 jaar anders zijn geweest zonder de mooie reizen die we samen hebben gemaakt. Elke keer weer kostte het nauwelijks moeite om even tot rust te komen, wat een toptijd! Paranimf **Rutger**, we zaten de laatste jaren allebei in hetzelfde schuitje, maar ondanks onze drukke schema's was er altijd wel ergens tijd om even lekker te klagen onder het genot van een biertje. We hebben in deze tijd veel leuke momenten samen gedeeld, met als hoogtepunt een lang weekend naar België; bedankt voor alles! Paranimf **Koen**, we hebben elkaar aan het begin van mijn PhD leren kennen tijdens een avondje 'Rikken' en zijn elkaar daarna niet meer uit het oog verloren. We hebben al heel wat leuke avondjes mee gemaakt, zeskamp Nuland, ons jaarlijks uitje 'Kwekfestijn', maar vooral ook de avondjes met een paar biertjes en slappe klets hielpen me de zware werkdag altijd weer te vergeten.

Natuurlijk wil ik ook mijn hele familie bedanken, maar in het speciaal **Pap, Mam, Twan, Mije,** en **Bas**. Bedankt voor alle steun, maar zeker ook voor het begrip voor de soms voor jullie onverwachte keuzes die ik in het verleden heb gemaakt om dit te kunnen bereiken. **Pap** en **Mam**, jullie hebben het altijd mogelijk voor mij gemaakt om mijn dromen na te streven, bedankt hiervoor! En broeders zoals we dat eigenlijk altijd doen, we pakken er nog eentje op!

Denise, je hebt nog een aardig graantje mee gepikt van mijn PhD tijd. Gedurende deze tijd werd je geduld met regelmaat op de proef gesteld, bedankt voor al je steun en begrip. Ik weet dat het niet makkelijk voor je was om iedere week weer naar Wageningen te komen, maar ik was er heel blij mee, een betere afleiding van mijn werk kon ik me niet wensen!

About the author

Curriculum Vitae

Rik Josephus Johannes van Erp was born on September 17, 1987 in 's Hertogenbosch, the Netherlands. After finishing his secondary school (Rodenborch, Rosmalen, The Netherlands) in 2005, he followed a BSc program in Animal Husbandry at Has Den Bosch ('s Hertogenbosch, the Netherlands). During his BSc internships, he worked for Cehave Landbouwbelang (Veghel, the Netherlands) and Hypor Genetics (Regina, Canada). Rik obtained his BSc degree in 2009 after which he started working as an Export Coordinator for TOPIGS (Vught, the Netherlands). In 2011, Rik continued his studies by following the MSc Animal Sciences at the Wageningen University. During this study, he followed a minor in Business and Management and a major specialization in Animal Nutrition. He finalized both his minor thesis entitled "Establishment of the degree of Standardization" and his major thesis entitled "The effect of replacing lactose from milk replacer by glucose, fructose or glycerol on the energy partitioning in milk-fed calves" in 2013. In 2014, he graduated for his MSc degree in Animal Sciences at the Wageningen University after which he took up the position as Researcher at Nutreco (Boxmeer, the Netherlands). While being employed by Nutreco, Rik became a PhD scholar at the Animal Nutrition Group of Wageningen University of which the results are presented in this thesis. Rik continuous to work at the R&D department of Nutreco as a Swine Researcher within the Swine Research Centre of Trouw Nutrition.

List of publications

Van Erp RJJ, de Vries S, Van Kempen TATG, Den Hartog LA, Gerrits WJJ: Feed intake patterns nor pig performance are affected by dietary resistant starch, despite marked differences in digestion. *Manuscript submitted for publication.*

Van Erp RJJ, Van Hees HMJ, Zijlstra RT, Van Kempen TATG, Van Klinken JB, Gerrits WJJ: Reduced feed intake, rather than increased energy losses explains variation in growth rates of normal birth weight piglets. *J Nutr* 2018, 148:1794-1803.

Van Erp RJJ, de Vries S, Van Kempen TATG, Gerrits WJJ: Pigs ferment enzymatically digestible starch when it is substituted for resistant starch. J Nutr 2019, nxz072, https://doi.org/10.1093/jn/nxz072.

Van Erp RJJ, de Vries S, Van Kempen TATG, Den Hartog LA, Gerrits WJJ: Circadian misalignment imposed by nocturnal feeding increases fat deposition in pigs. *Manuscript submitted for publication.*

Training and supervision plan[1]

	Year
The Basic Package (2 ECTS[2])	
WIAS Introduction Course, Wageningen, the Netherlands	2015
Philosophy and Ethics of Science Course, Wageningen, the Netherlands	2015
Disciplinary Competences (16 ECTS)	
Quality of Protein in Animal Diets, Wageningen, the Netherlands	2015
Writing PhD Research Proposal	2016
Course on Indirect Calorimetry, Krakow, Poland	2016
Carbohydrates in Pigs, Viborg, Denmark	2017
Introduction to Laboratory Animal Science, Wageningen, the Netherlands	2018
Mixed Linear Models, Wageningen	2018
Professional Competences (4 ECTS)	
Scientific Writing, Wageningen, the Netherlands	2016
Project Management, Amersfoort, the Netherlands	2017
Techniques for Writing and Presenting, Wageningen, the Netherlands	2018
Presentation Skills (4 ECTS)	
International Symposium on Energy and Protein Metabolism and Nutrition, Krakow, Poland (Poster presentation)	2016
International Symposium on Energy and Protein Metabolism and Nutrition, Krakow, Poland (Oral presentation)	2016
Course on Indirect Calorimetry, Krakow, Poland (Short lecture)	2016
European Starch Round Table, Lille, France (Oral presentation)	2016
Digestive Physiology in Pigs, Brisbane, Australia (Poster presentation)	2018
Digestive Physiology in Pigs, Brisbane, Australia (Oral presentation)	2018
WIAS Science Day, Wageningen, The Netherlands (Oral presentation)	2018
Teaching Competences (6 ECTS)	
Supervising MSc students (5x)	2015-2019
Practical Supervision	
Introduction to Animal Science	2016, 2017
Introduction to Animal Nutrition	2017

Total 32 ECTS

[1]Completed in fulfilment of the requirements for the education certificate of the Wageningen Institute of Animal Sciences (WIAS)
[2]One ECTS credit equals a study load of 28 hours