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Role of starch chemistry in the kinetics of nutrient absorption, endocrine profile, and intestinal health in swine

by

Prajwal Raj Regmi

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DEDICATION

I dedicate this thesis to my loving wife Bina and daughters Sezal and Serene, who have given me encouragement and support throughout the Ph. D. program.

ABSTRACT

Starch is a major source of energy for monogastric species. Although starch digestibility is understood, the role of starch chemistry on nutrient absorption, endocrine function and gut health is not. Therefore, the objective was to characterize the role of starches with a range in amylose content and rate of in vitro digestion on the kinetics of nutrient absorption, endocrine profile, and gut microbial profile in pigs. Four high (70%) starch diets differing in amylose contents (0, 19.6, 28.4 and 63.2%) and rates of in vitro digestibility (1.06; S2, 0.73; S3, 0.38 and S4, 0.22%/min; rapidly to slowly digestible starches) were formulated. In study 1, four portal vein-catheterized pigs were fed the starch diets and blood samples were collected from the portal vein, carotid artery and simultaneous blood flow was measured until 12 hour after feeding. In vitro rates of glucose release corrected for gastric emptying was strongly related (R^2 = 0.95) to the kinetics of portal glucose absorption. Slowly digestible starch decreased glucose absorption and secretion of insulin and glucose-dependent insulinotropic polypeptide (GIP) but increased butyrate absorption and plasma betaine concentration. In study 2, fecal, ileal digesta, and urine samples were collected from eight ileal-canulated pigs fed the same diets. Slowly digestible starch decreased starch digestibility, increased total short chain fatty acids (SCFA) and butyrate production and selectively increased the population of bifidobacteria in the gut. In addition, mineral digestion and absorption in the large intestine compensated lower absorption of Ca, P, Na, and Fe in the small intestine of pigs fed slowly digestible starch. In summary, slowly digestible starch is energetically

less efficient compared to rapidly digestible starch and the starch reduces glucose absorption and insulin secretion and increases the production of butyrate and the population of bifidobacteria. However, substantial changes of starch chemistry (at least $\geq$ 40% amylose content and $\leq$ 0.36%/min of maximum in vitro digestion rate) were required to have potentially beneficial changes in pigs.

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LIST OF ABBREVIATIONS

^{1}H	Proton
$½T_{Cmax}$	Duration for reaching half of peak concentration starting from peak concentration
AOAC	American association of cereals chemists
AUC	Area under the curve
BCFA	Branched-chain fatty acid
BW	Body weight
C	Shape parameter of in vitro glucose release
CCAC	Canadian council on animal care
C_{max}	Peak concentration
CP	Crude protein
$C_{plateau}$	C, shape parameter, adjusted for plateau effects
CV	Coefficient of variance
DGGE	Denaturing gradient gel electrophoresis
DE	Digestible energy
DM	Dry matter
GC	Gas chromatography
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
HDL	High density lipoprotein
K	Rate of glucose release

$K_{plateau}$	K, rate parameter, adjusted for plateau effects
LDL	Low density lipoprotein
NMR	Nuclear magnetic resonance
NPA	Net portal appearance
PC	Principal component
PDV	Portal drained viscera
PLS-DA	Partial least square - discriminant analysis
qPCR	Quantitative polymerase chain reaction
RDS	Rapidly digestible starch fraction
RIA	Radioimmunoassay
RMSE	Root mean square error
RS	Resistant starch
S1	Rapidly digestible starch diet
S2	Moderately rapidly digestible starch diet
S3	Moderately slowly digestible starch diet
S4	Slowly digestible starch diet
SCFA	Short-chain fatty acids
SEM	Standard error of mean
SDS	Slowly digestible starch
T_{max}	Time to reach peak concentration

Chapter 1. The role of starch chemistry in nutrient digestibility and absorption, endocrine responses, and gut microbial profile in monogastric species: A review

1.1 Introduction

Cereal grains and legume seeds are major sources of starch in diets for humans and monogastric animals. Starch is digested in the intestine before being utilized by the body for different functions including energy production. The rate and extent of digestion of starch is affected by its physicochemical properties such as starch chemistry (Franco et al., 1992; Morita et al., 2007), particle size (Mahasukhonthachat et al., 2010), processing methods (Jenkins et al., 1982), and association with other compounds such as lipids, protein, fiber, mineral, and anti-nutritional factors (Thorne et al., 1983; Thompson et al., 1987; Cui and Oates, 1999; Blennow et al., 2000). Among starch chemistry, ratio of amylose and amylopectin, ratio of crystalline and amorphous region (crystallinity), and size of starch granules are major characteristics that affect starch digestion.

Starch is digested mainly by pancreatic α-amylase in the small intestine and undigested starch is available for microbial fermentation mainly in the large intestine. Small intestinal starch digestibility affects glucose and insulin responses that are associated with the risk of metabolic diseases such as diabetes in humans and with feed efficiency, growth rate and lean vs. fat deposition in food animals. Availability of starch for microbial fermentation may increase the population of

beneficial microflora such as bifidobacteria and production of short chain fatty acids (SCFA) including butyrate. These properties are important for a healthy gut, because they reduce risks of diseases such as colon cancer and inflammatory bowel disease (Scheppach et al., 2001; Bauer-Marinovic et al., 2006). Starch may also play a role in reducing risk of obesity by beneficially affecting indicators of fat metabolism such as plasma cholesterol and triglyceride (Seal et al., 2003; Wolever, 2003; Ells et al., 2005). Starches with a high amylose and resistant starch (RS) content are currently included in several commercial food applications for their health benefits (Taggart, 2004). However, it is not clear a) whether the added amylose is truly beneficial for consumers and b) whether the amount that is included in food is sufficient to bring beneficial changes.

Starch has been the subject of research over many years. Several reviews on different aspects of starch are available including reviews on physico-chemical characteristics (Parker and Ring, 2001; Sajilata et al., 2006; Copeland et al., 2009; Hoover et al., 2010a) and factors affecting digestibility (Wiseman, 2006; Copeland et al., 2009; Dona et al., 2010), nutritive value (Tapsell, 2004; Svihus et al., 2005) and health (Nugent, 2005; Lehmann and Robin, 2007; Fuentes-Zaragoza et al., 2010). However, concise information relating starch characteristics to starch digestibility and subsequent metabolic responses is lacking. Moreover, inconsistencies exist with regards to the role of starch on physiological responses such as nutrient absorption, hormone secretion, fat metabolism, and microbial fermentation. Finally, starch chemistry effects are

often confounded with effects of associated compounds such as fiber and protein. The objective of this review was to compile research publications on the role of starch chemistry on nutrient digestibility and absorption, hormone profile and gut microbial fermentation in humans and monogastrics and provide conclusive results based on findings of previous studies. Among the starch chemistry variables, amylose and amylopectin ratio, crystallinity, and granule size were reviewed. Effects of starch particle size, processing, and starch association with other compounds were beyond the scope of the review. A comprehensive search of the literature was first conducted to locate appropriate studies to be included in this review. This was performed using major search engines such as Pubmed, Sciencedirect, Agricola, High wire press, CAB, Wiley interscience, EBSCOhost for the review. For consistency of starch terminologies, starches with high and low amylose, high and low RS, and native legume and native cereal starches were considered slowly digestible (SDS) and rapidly digested starches (RDS), respectively.

Furthermore, a meta-analysis approach (Comprehensive Meta Analysis Version 2.0, Biostat Inc., Englewood, NJ, USA) was used when at least 5 studies reported the effect of starch chemistry on a variable. Studies with insufficient data for effect size calculations (e.g., with means but no standard deviations or no standard error) were excluded. Data from the meta-analysis were presented as mean ± SEM. When the standard deviation was reported in the original article, the value of the SEM was calculated. In some studies with more than 2 treatments, more

than one comparison was made. For the analyses, $P < 0.05$ was considered significant. A meta-analysis gives weight to a study based on quality and quantity of information such as sample size, variance, and frequency in the study; whereas, traditional review approach treats all studies equally regardless of the quality and quantity of the information presented. Because of the quantitative nature of a meta-analytic review, its application allows for a more objective analysis and less bias than the more traditional review process (Egger and Smith, 1997).

1.2 Starch composition and effects on digestibility

Starch is present in the form of granules within cell walls and embedded in the protein matrix in the endosperm of cereal grains and cotyledons of legume seeds. Starch granules contain two main structural components: amylose and amylopectin polymers. Amylose is linear or slightly branched, with up to 6,000 glucose units linked by α-D-(1-4) bonds (Takeda et al., 1989). Amylopectin is a larger highly-branched molecule with an average of a million glucose units linked by α-D-(1-4) and α-D-(1-6) chains (Parker and Ring, 2001). Amylopectin is organized in a ring-like structure in the granules that determine the relative proportion of crystalline region with densely packed glucose units; whereas, the amorphous region is formed by loosely packed glucose units (**Figure 1.1**). Although amylopectin is the predominant crystalline component in starch, amylose in high amylose starches may form double helices that result in crystalline regions (Tester et al., 2004).

In addition to amylose and amylopectin glucose polymers, minerals, protein, and fats are also detected in starch granules (**Table 1.1**). Among the compounds, lipids can form complexes with amylose and proteins can form matrixes around starch granules (Tester et al., 2004); both compounds can thereby affect starch digestibility. Moreover, moisture, which may be related to the granular architecture, can also influence starch digestibility (Tester et al., 2004).

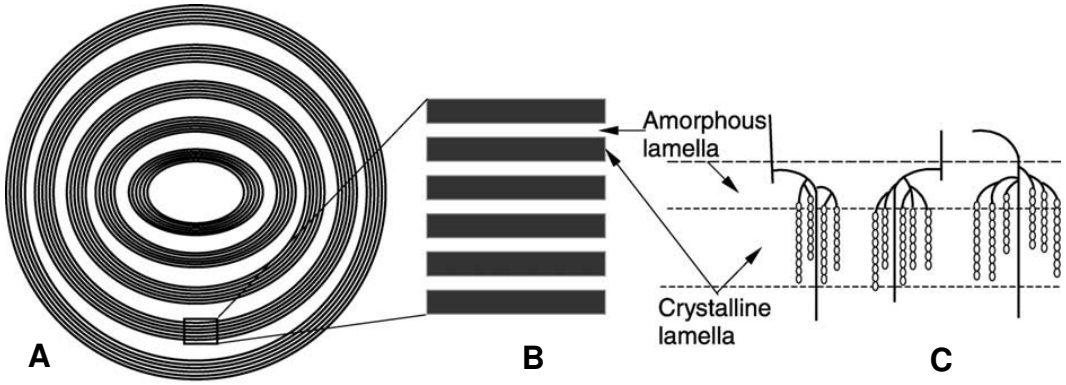

Figure 1.1 Diagrammatic representation of crystalline and amorphous structure of a starch granule. (**A**) Stacks of crystalline lamellae separated by amorphous rings. (**B**) Magnified view of the amorphous and crystalline regions. (**C**) Double helical structures formed by adjacent chains of amylopectin give rise to crystalline lamellae. Branching points constitute the amorphous regions (Source: (Tester et al., 2004).

Table 1.1 Composition of starch granules

Characteristic	Minimum	Maximum	References
Moisture	9.2	19.0	(Hoover et al., 2010a)
Protein	0.07	0.43	(Li et al., 2001)
Nitrogen, %	0.001	0.43	(Hoover and Senanayake, 1996; Hoover et al., 2010a)
Free lipids	0.11	0.29	(Li et al., 2001)
Bound lipids	0.27	1.32	(Li et al., 2001)
Total lipids, %	0.01	1.33	(Hoover et al., 2010a)
Ash	0.03	0.42	(Li et al., 2001)
Phosphorous	0.05	0.75	(Vasanthan et al., 1999; Yusuph et al., 2003)
β-glucan	Not detected	0.06	(Li et al., 2001)

1.2.1 Starch granule

Starch granules range in size (0.4 to 100 μm in diameter; **Table 1.2**), shape (round, lenticular, polygonal), size distribution (uni- or bi-modal), and occur as simple (isolated individual), granule or granule clusters (compound) (Tester et al., 2004). Previously, starch digestibility was considered higher for smaller granules probably due to a greater surface area per unit of enzyme (Manelius and Bertoft, 1996). For small and large granules separated from wheat starch, small granules had a greater in vitro digestion rate until 4 h, while large granules had a greater rate of digestion after 4 h (Salman et al., 2009). This difference indicates that at later stage of digestion, the rate of digestion is less influenced by granule size and more influenced by volume and internal structure of starch granules.

1.2.2 Amylose content and amylopectin architecture

Amylose content in starch can differ among starches from different botanical origin. Starch containing less than 15% amylose is known as 'waxy', from 20 to 35% amylose as 'regular' and starch containing more than 40% amylose as 'high amylose' starch. The amylose content of starch ranged from 0 to 94.6% for cereals and from 13 to 88% for legumes (**Table 1.2**).

Table 1.2 Granule size, amylose content and crystallinity of starches from cereals, legumes, and potato

Starch source	Granule diameter, μm	Amylose, %	Crystallinity, % [a]	Reference
Cereals				
Rice	3 – 150	0 – 40	38 – 51	(Juliano, 1992; Hu et al., 2004; Tester et al., 2004)
Barley	2 – 25	0 – 70	20 – 37	(You and Izydorczyk, 2002; Bird et al., 2004; Tester et al., 2004)
Wheat	<1 – 53	<1 – 38	36 – 39	(Kiribuchi-Otobe et al., 1997; Yamamori et al., 2000; Tester et al., 2004; Dai et al., 2008)
Corn	2 – 30	0 – 95	17 – 48	(Deng et al., 20010; Tester et al., 2004; Kurakake et al., 2009)
Oat	3 – 105	20 – 25	28 – 37	(Vasanthan et al., 1999; Hoover et al., 2003; Tester et al., 2004)
Legumes				
Smooth pea	5 – 50	13 – 49	17 – 31	(Jenkins and Donald, 1995; Tester et al., 2004; Hoover et al., 2010a)
Wrinkled pea	5 – 37	60 – 88	18	(Colonna and Mercier, 1985; Hoover et al., 2010a)
Field pea	5 – 12	48 – 50	20 – 25	(Ratnayake et al., 2001)
Cowpea	3 – 64	26 – 33		(Hoover et al., 2010a)
Chickpea	9 – 30	30 – 40	18 – 35	(Hughes et al., 2009; Hoover et al., 2010a)
Lentil	6 – 37	24 – 32	19 – 32	(Hoover et al., 2010a)
Black bean	7 – 55	27 – 39	17 – 33	(Jenkins and Donald, 1995; Hoover et al., 2010a)
Kidney bean	16 – 60	42	28 – 30	(Hoover et al., 2010a)
Faba bean	9 – 48	17 – 42		(Hoover et al., 2010a)
Potato	5 – 100	<1 – 95	24 – 40	(Ortega-Ojeda et al., 2004; Tester et al., 2004)

[a] Crystalinity can be of types A, B and C based on the botanical origin of the starch.

The digestion of starch is inversely related to its amylose content (Topping and Clifton, 2001; Svihus et al., 2005). This relation may be due to decreased accessibility of α-amylase to amylose polymers resulting from greater intermolecular hydrogen bonding in these polymers (McGrane et al., 2004) and the formation of complexes between amylose and surface compounds such as fatty acids (Cui and Oates, 1999). In high amylose starch, amylose polymers can retrograde among themselves further decreasing the enzyme accebility (REF). In addition to amylose content, length of the amylopectin chain affects the kinetics of starch digestibility. The extent of digestion is related positively with short amylopectin chains from 30 min to 4 h; negatively with medium and long amylopectin chains from 30 min to 1 h; and positively with medium length amylopectin chains from 4 to 24 h (Salman et al., 2009).

Amylose content and starch granule size interact to influence starch digestion. Starch digestion was slower for high amylose varieties of barley even if these contained a higher proportion of smaller starch granules than low amylose varieties. This difference indicates that amylose content has greater influence on starch digestibility than granule size (Rudi et al., 2006).

1.2.3 Crystallinity

Crystallinity of starch can range from 17 to 51% (**Table 1.2**). Starch crystallinity can be divided into types A, B, and C based on packing of double helices of starch polymers, mainly amylopectin, and number of water molecules (**Figure 1.2**). Type A crystallinity is characterized by relatively compact double helical arrangements with a

low water content. Type B has a more open structure with a hydrated helical core. Type C crystallinity combines type A and B. Type A crystallinity is present in cereal starches, type B in tubers and high amylose starches; and type C in legume starches.

Higher crystallinity can decrease starch digestibility because starch digesting enzymes such as α-amylase have a lower accessibility to crystalline regions of starch granules than amorphous regions (Kim et al., 2008). In addition, starches with different crystallinity pattern have unique digestion properties. For instance, starch granules with type A crystallinity are digested from the inside (MacGregor and Ballance, 1980) which that might be the reason for the sigmoidal digestion pattern of starches from cereal grains.

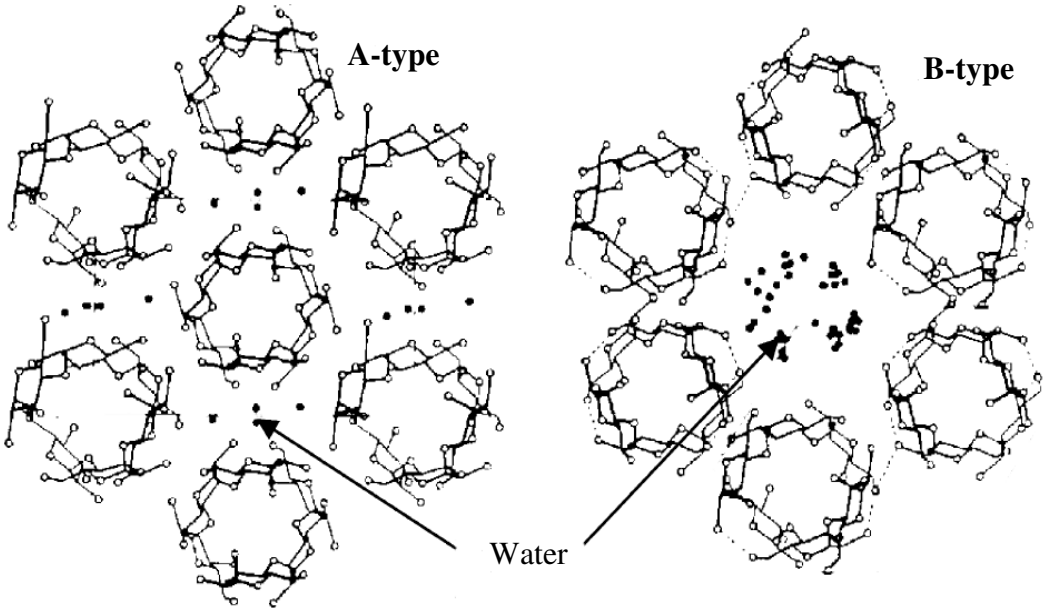

Figure 1.2 Diagrammatic representation of A- and B-type crystallinity pattern in starch granules (Source: Tester et al., 2004).

1.3 Starch characterization to describe in vivo starch effects

Several characteristics of starches are used to describe the effects of starch types on in vivo responses. For example, amylose content, botanical origin, processing, and relative proportion of resistant starch (RS) content have been used. Among these characteristics, amylose and RS content are considered good indicators of in vivo starch effects. The RS content of starch is defined as the starch fraction not digested within 120 min (Englyst et al., 1992; Englyst et al., 1999) and is determined by an in vitro assay (Englyst et al., 2000).

Generally, starch with a higher amylose and RS content has a lower starch digestion and thus results in lower postprandial blood glucose than starches with a lower amylose and RS content. However, these relationships are not always consistent. For instance, rice starch containing 22% amylose had higher starch digestion rates and glycemic responses than rice starch containing 15% amylose (Rao, 1971) and starch with similar amylose content had different rate of in vitro starch digestibility (Salman et al., 2009). Therefore, although amylose content is an important indicator of starch digestibility, variations in other aspects of starch chemistry such as granule size and the molecular architecture of amylopectin introduce uncertainty into the prediction of in vivo starch effects from amylose content alone (Copeland et al., 2009). In addition, a greater RS content (11 vs. 0%) did not reduce postprandial glucose level in peripheral circulation (Noah et al., 2000; Higgins et al., 2004). Enzymatic starch digestion in vivo can continue beyond 120 min (McCleary et al., 2002) indicating the RS determined using the current in vitro approach has poor biological relevance and may not be a valid

indicator of in vivo digestibility (Schulz et al., 1993). In a study, approximately 50% of RS was found to be digested in small intestine in human (Vonk et al., 2000). Therefore, amylose and RS content alone may not be considered as starch characteristics that can sufficiently explain starch effects in vivo (Panlasigui et al., 1991).

Starch characteristics that accurately predict in vivo physiological responses are required to avoid inconsistencies among studies. Starch digestion in the small intestine is reflected by postprandial blood glucose profile although minor portion may be used by enterocytes during luminal glucose transport. Thus, postprandial blood glucoe profile is the direct link between in vivo responses and characteristics of starches. Therefore, for studies involving starch effects on in vivo response, the starch characteristics that have consistent effects on in vivo glucose response are important to consider. Starch digestion in the small intestine is not solely affected by a single, distinct chemical entity but by a combination of physical and chemical characteristics. In this review, starch diets were considered either SDS or RDS diets based on relative content of amylose, RS, in vitro digestibility or source of starch.

1.4 Starch chemistry effects on physiological responses

Starch chemistry can affect physiological responses by modulating metabolic and endocrine stimuli in the body and the profile of microbiota and fermentation products in the gut lumen (**Figure 1.3**). These physiological responses from starch can modulate the risk of human diseases related to carbohydrate metabolism such

as diabetes, obesity, and cardiovascular disease. In addition, microbial profile and fermentation in the gut can modulate the risk of gut diseases (Mathers and Daly, 1998). In animals, kinetics of starch digestion can influence feed efficiency, growth rate and body fat content (Weurding et al., 2001; van Kempen et al., 2007).

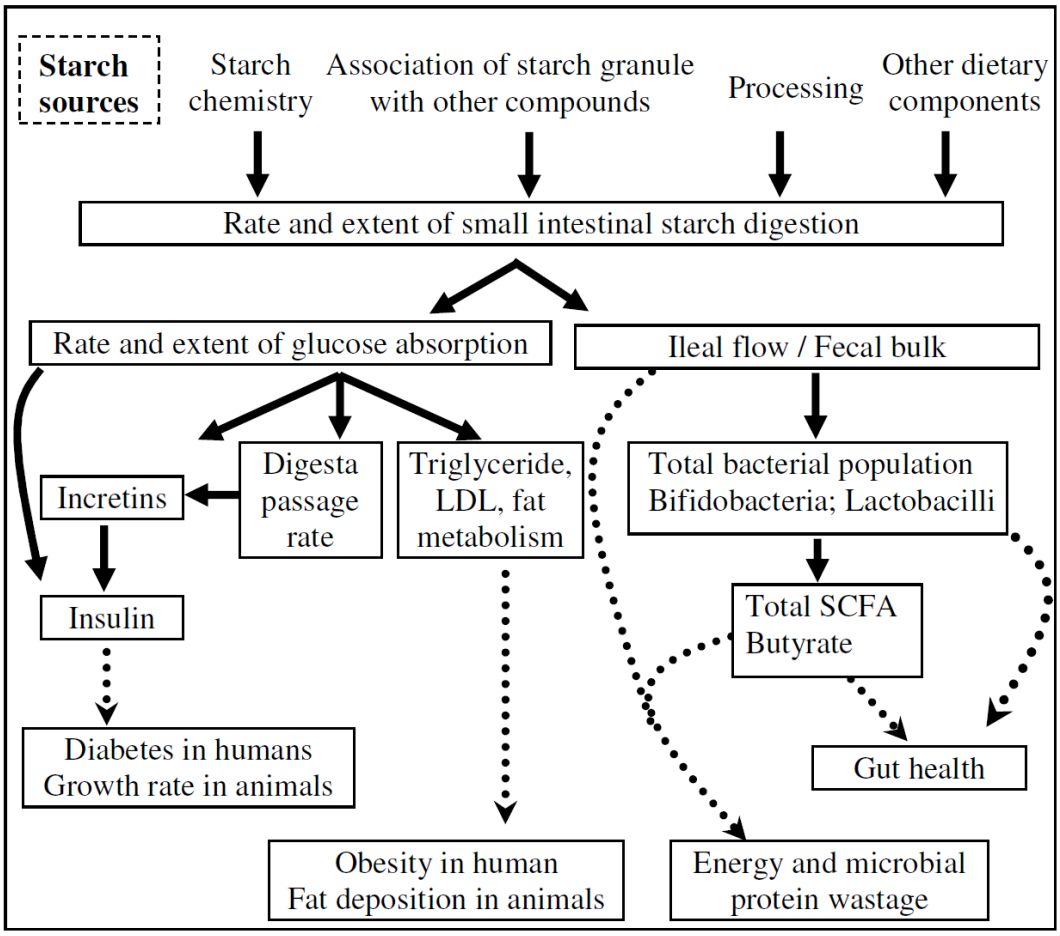

Figure 1.3 Overview of role of starch on physiological response and relation with human health and animal performance; solid arrows indicate direct effects and dashed arrows indicate relationships.
(Source: (Jenkins et al., 1982; Thorne et al., 1983; Thompson et al., 1987; Franco et al., 1992; Cui and Oates, 1999; Blennow et al., 2000; Parker and Ring, 2001; Scheppach et al., 2001; Seal et al., 2003; Wolever, 2003; Tapsell, 2004; Ells et al., 2005; Nugent, 2005; Svihus et al., 2005; Bauer-Marinovic et al., 2006; Sajilata et al., 2006; Wiseman, 2006; Lehmann and Robin, 2007; Morita et al., 2007; Copeland et al., 2009; Dona et al., 2010; Fuentes-Zaragoza et al., 2010; Hoover et al., 2010b; Mahasukhonthachat et al., 2010).

1.4.1 Digestibility and fecal output

In vitro and in vivo digestibility of different starch types have been reported (**Table 1.3**). Starches originated from a particular crop can have a wide variability in chemistry, and thus, in vitro and in vivo digestibility. For instance, 0 to 20 min and 20 to 120 min in vitro digestibility and RS content of corn starch can range from 6 to 28, 18 to 54 and 17 to 62%, respectively; and ileal digestibility of corn starch in pigs and humans can range from 47 to 96% (**Table 1.3**). In addition, SDS reduces gastric emptying (Borgida and Laplace, 1977) probably due to the enteroglucagon stimulating effect of SCFA (Gee et al., 1996). Enteroglucagons such as oxyntomodulin and glicentin are peptide secreted by intestinal L cells in response to higher SCFA and reduce gastric emptying in monogastric species (Schjoldager et al., 1989). Slow gastric emptying reduces digesta passage rate and increases contact between digestive enzymes and starch, and thus, may increase digestion (Borgida and Laplace, 1977).

Starch digestion affects digestibility of other nutrients present in monogastric diets. Slowly digestible starch decreased ileal digestibility of total carbohydrate and non-starch polysaccharide (NSP) but increased total tract digestibility of total carbohydrate and NSP (Sun et al., 2006). Similarly, SDS can decrease ileal and total tract digestibility of crude protein (CP) (Kishida et al., 2001; Sun et al., 2006) and may decrease energy absorption (de Deckere et al., 1995). Slowly digestible starch either decreased (de Deckere et al., 1995) or did not affect (Noakes et al., 1996) total tract digestibility of fat. Reasons for decreased apparent ileal digestibility of CP could be: a) lower enzymatic digestion of protein due to lower enzyme to substrate ratio or inaccessibility of enzyme to protein entrapped

in undigested starch granules, and b) greater endogenous CP flow. In the large intestine, greater microbial fermentation and microbial protein synthesis by bacteria by using SDS as a substrate might increase carbohydrate digestibility and reduce protein digestibility.

Table 1.3 In vitro and vivo digestibility of starches from cereals, legumes and potato.

Starch source	In vitro			In vivo		Reference
	0 to 20 min	20 to 120 min	RS	Ileal	Total tract	
Cereals						
Rice	20 – 29	47 – 68	3 – 34	100	100	(van Kempen, T.A.T.G. personal communication)
Barley	16	72	13	93	100	(Sun et al., 2006)
Corn	6 – 28	18 – 54	17 – 62	47 – 96	99	(Ferguson et al., 2000; Wachters-Hagedoorn et al., 2006; Bird et al., 2007)
Legumes						
Smooth pea	8 – 24	34 – 59	8 – 58	78 – 100	99 – 100	(Berggren et al., 1995; Sun et al., 2006; Hoover et al., 2010a)
Field pea	–	18 – 22*	78 – 82	–	–	(Ratnayake et al., 2001)
Chickpea	11 – 30	35 – 60	8 – 41	–	–	(Hughes et al., 2009; Hoover et al., 2010a)
Lentil	5 – 17	30 – 62	13 – 65	–	–	(Hoover et al., 2010a)
Kidney bean	12	66	17	–	–	(Hoover et al., 2010a)
Potato	2 – 7	14 – 26	65 – 73	–	–	(Raben et al., 1994; Ferguson et al., 2000) (Liu et al., 2007)

*RDS + SDS, values are not available for wheat, oat, wrinkled pea, cow pea, black bean, faba bean.

Fecal bulk is an indicator of gut health in humans because it can influence normal bowel movement and microbial activity in the distal gut. Although the bulk-forming property of SDS was reported to be low (<1.0 mL/g; approximately one-fifth of cellulose; (Takeda and Kiriyama, 1979), starch digestibility affects digesta flow into the large intestine and thus, fecal bulk. A meta-analysis of 22 research studies indicated that SDS increased ($P < 0.001$) fecal bulk compared to RDS (**Figure 1.4**). In the analysis, SDS increased fecal bulk in 10 studies and had no effect on fecal bulk in 12 studies. In diets containing at least 40% starch, fecal bulk was greater for high amylose than waxy starch group but did not differ from normal amylose. In addition, an increase of 14% in RS failed to yield greater fecal bulk. The analysis indicated that starch chemistry needs to be drastically different to yield greater fecal bulk in monogastric species.

1.4.2 Feed efficiency and body weight gain

Starch chemistry may affect feed efficiency and body weight gain in monogastric species. However, previous studies did not show consistent effect of high amylose or RS on feed efficiency and body weight gain. Feed efficiency (gain/feed ratio) was either decreased (de Deckere et al., 1995; Kim et al., 2003) or unaffected by SDS (de Deckere et al., 1993; Verbeek et al., 1995; Kishida et al., 2001; Bird et al., 2007). Starch containing high amylose (more than 40%) or high RS did not affect feed efficiency compared with waxy starch (less than 15%) or starch with very low amount of RS (for instance < 0.8 g in rat diet), but had lower feed efficiency compared with starch containing regular amylose (20 to 35%) or moderate level of RS (**Table 1.3**). This observation indicated that perhaps sustained and moderate level of starch digestion in starch containing regular

amylose or moderate level of RS improve feed efficiency compared to very rapid and very slow starch digestion in starch containing very high or very low amylose or RS.

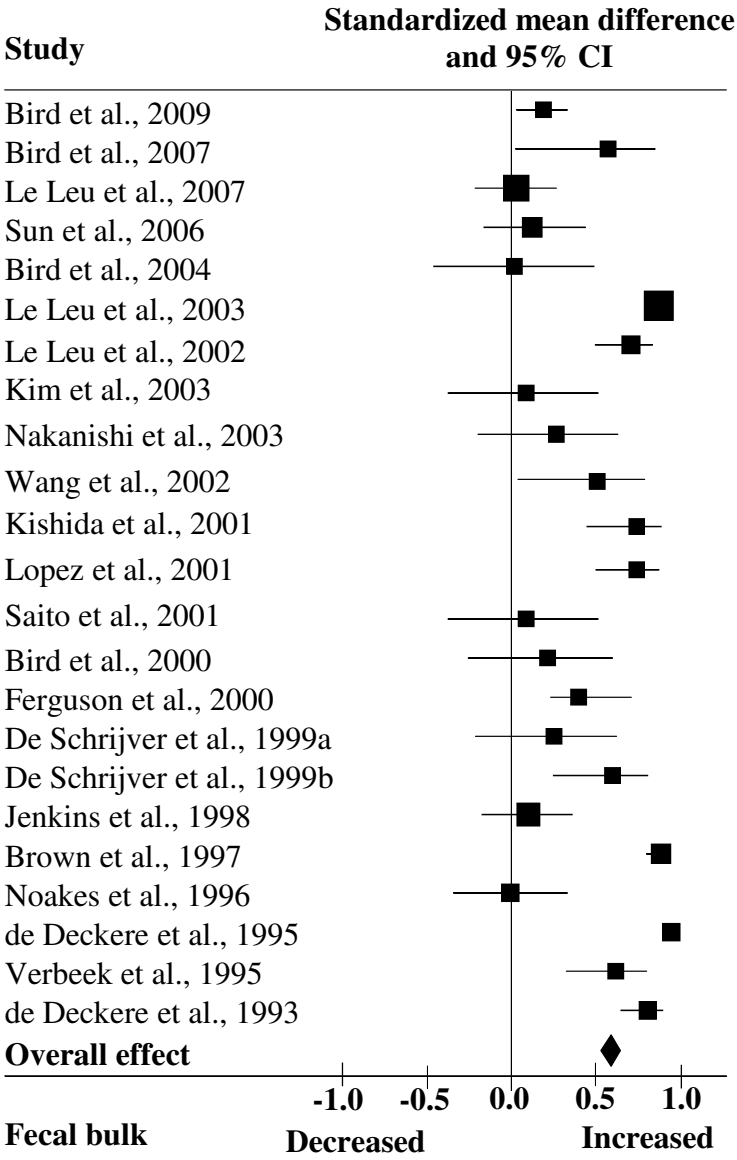

Figure 1.4 Meta-analysis of effect of slowly digestible starch on fecal bulk; boxes and horizontal lines indicate individual study effects and confidence interval; the size of the box is proportional to the relative weight of the study; diamond indicates the overall effect; vertical line drawn at "zero" indicates no effect; CI, confidence interval; test for overall effect: Z = 7.05 $P < 0.001$.

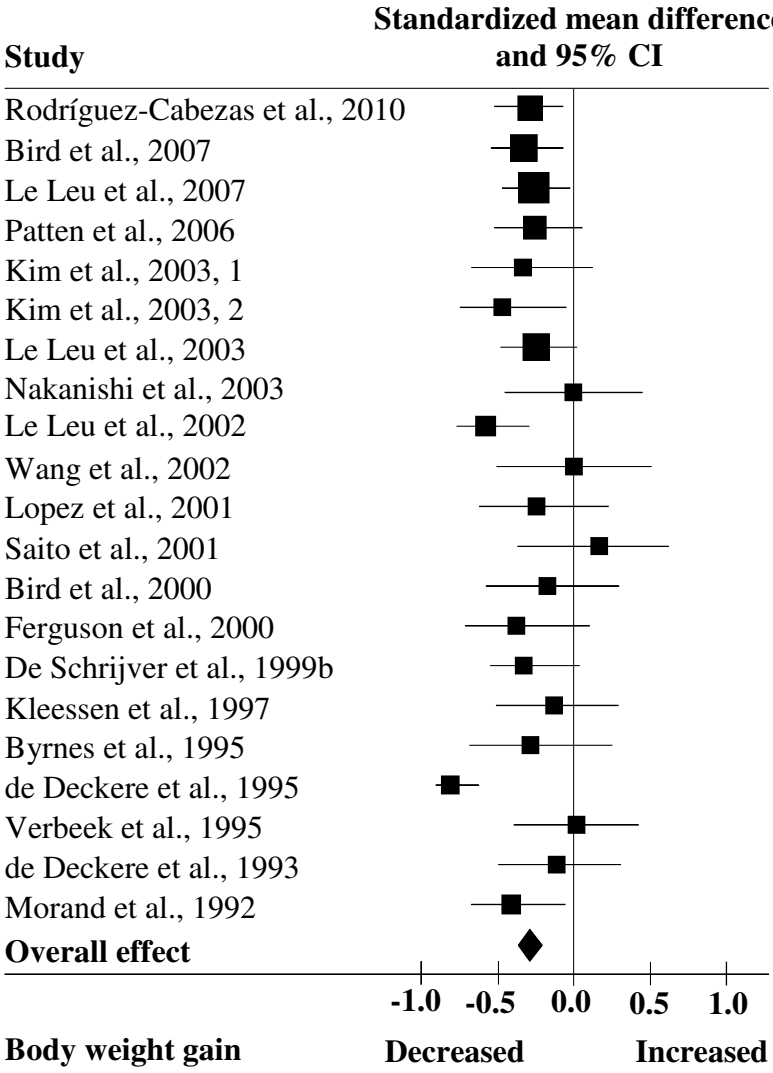

Figure 1.5 Meta-analysis of effect slowly digestible starch on body weight gain; boxes and horizontal lines indicate individual study effects and confidence interval; the size of the box is proportional to the relative weight of the study; diamond indicates the overall effect; vertical line drawn at "zero" indicates no effect; CI, confidence interval; test for overall effect: Z = -6.32, $P < 0.001$.

Meta-analysis of 20 research studies with a total of 21 comparisons showed the SDS decreased ($P < 0.001$) body weight gain in monogastric species (**Figure 1.5**). In the analysis, 7 comparisons indicated lower body weight gain for SDS compared to RDS and in 14 comparisons SDS did not affect body weight gain. These studies indicate that starch containing as low as 10% greater RS sometimes

lowered body weight gain; while starch containing up to 13.6% greater RS did not lower body weight gain. This finding clearly shows that RS alone is not an accurate predictor of starch effect on body weight gain.

1.4.3 Intestinal morphology

Starch chemistry can affect proliferation and differentiation of intestinal epithelium (Roediger, 1990; Cummings and Englyst, 1991; Scheppach, 1994; Silvi et al., 1999) that may eventually affect the gut mass. Previously, SDS either increased (de Deckere et al., 1995; Bird et al., 2007) or had no effect on the length of large intestine (de Deckere et al., 1993; Kim et al., 2003). However, SDS consistently increased the weight of small intestine (de Deckere et al., 1993; de Deckere et al., 1995), cecum (de Deckere et al., 1995; Silvi et al., 1999; Lopez et al., 2001; Le Leu et al., 2003; Bird et al., 2007), and colon (Bird et al., 2007) except in one study where SDS did not increase cecal length (Kim et al., 2003). Starch with a high amylose or RS content increased the gut mass compared with starch containing low amylose or RS but did not affect gut mass compared with starch containing regular amylose or moderate level of RS (**Table 1.4**). This observation parallels with the starch effects on digesta mass and supports previous findings that digesta mass is a major predictor (R^2 = 0·83; (Bird et al., 2007) of gut mass.

Table 1.4 Studies describing starch effects on the profile of nutrients, hormones and gut microbiota reviewed

Diets and duration	Species (n)	Treatments	Reference
2 g RS day in water	Rat (*n* = 20)	2 vs. 0 g RS/day	(Rodríguez-Cabezas et al., 2010)
70% starch, 14% casein, 7.4% fish meal, 4% cellulose, 1% oil for 7 d	Pig (*n* = 4)	1.06 vs. 0.73 vs. 0.38 vs. 0.22 rate of in vitro starch digestion %/min	(van Kempen et al., 2010)
48% of diet as starch, 40g of fiber/d from wheat bran for 7d	Pig (*n* = 4)	Starch as 85 vs. 0% amylose	(Bird et al., 2009)
39% starch, 3% oil for 21 d	Pig (*n* = 6)	94.6 vs. 18.2 vs. 17.6 vs. 0 % amylose	(Deng et al., 20010)
44 - 45% starch, 3% oil for 7 d	Pig (*n* = 4)	High amylose corn vs. normal corn vs. brown rice vs. waxy rice	(Li et al., 2008)
2 g starch/kg body wt., 1 meal	Human (*n* = 21)	67.7 vs. 60.5% RS	(Bhattacharya et al., 2007)
54% starch, 20% lipid for 21 d	Human (*n* =24)	85 vs. 0% amylose	(Bird et al., 2007)
Standard rat diet 46.2 – 57.2% starch for 28 d	Rat (*n* = 30)	10 vs. 0% RS	(Le Leu et al., 2007)
25 – 35% starch for 96 d	Pig (*n* = 8)	Raw potato vs. corn starch	(Nofrarias et al., 2007)
1 g carbohydrate/kg body wt., 1 meal	Human (*n* =);	5.06 vs. 2.57 vs. 0.71 g RS/100 g muffin	(Behall et al., 2006)
50% starch, 20% casein, 10% sucrose 7% oil, 5% fiber for 28 d	Rat (*n* = 8)	50% corn starch vs. 25% corn starch and 25% high amylose starch	(Chang et al., 2006)
45-55% starch, 20% casein, 0-10% cellulose, 10% sucrose, 10% oil for 41 d	Rat (*n* = 5);	High amylose vs. regular corn starch	(Patten et al., 2006)
40% starch, 4% fat for 15 d	Pig (*n* = 18)	17.1 SDS, 21.9%RS vs. 45.6% SDS, 8.1% RS	(Sun et al., 2006)
50 g glucose equivalent starch, 1 meal	Human (*n* = 7)	28% RDS and 45.3% SDS vs. 89% RDS and 6.8%SDS	(Wachters-Hagedoorn et al., 2006)
1 g carbohydrate/kg body wt., 1 meal	Human (*n* = 24)	70 vs. 30% amylose	(Behall and Scholfield, 2005)
75 g starch diet for 14 d	Human (*n* = 10)	46.8% RDS and 45.5% SDS vs. 95.5% RDS	(Ells et al., 2005)
60% carbohydrate, 20% protein and 8% fat for 21 d	Pig (*n* = 7)	High amylose barley vs. regular barley whole meal	(Bird et al., 2004)
55% carbohydrate; 17% fat, 1 meal	Human (*n* = 12)	10.7 vs. 5.4 vs. 2.7 vs. 0% RS	(Higgins et al., 2004)

Table 1.4 contd…

Diets and duration	Species (n)	Treatments	Reference
65.3 % corn starch and 25% casein diet for 14 d	Rat (*n* = 6);	30 vs. 20 vs. 0% amylose starch	(Morita et al., 2004)
53% starch, 7% oil for 21 d	Diabetic rat (*n* = 8)	16.1% corn RS vs. 16.1% rice RS vs. 2.5% corn RS	(Kim et al., 2003)
47% starch, 1.8% fat for 28 d	Rat (n= 24)	18.5, 12.4, 6.2 vs. 0% RS	(Le Leu et al., 2003)
20% diet as high amylose or regular starch for 21 d	Rat (*n* = 8)	70% vs. regular amylose	(Nakanishi et al., 2003)
50 g starch/person, 1 meal	Human (*n* = 8)	68 vs. 96 % 360 min-in vitro digestibility	(Seal et al., 2003)
55% carbohydrate, 30% fat, 1 meal	Human (*n* = 24)	70 vs. 60 vs. 50 vs. 40 vs. 30% amylose	(Behall and Hallfrisch, 2002)
Standard rat diet for 21 d	Rat (*n* = 12)	11.3 vs. 0% RS	(Le Leu et al., 2002)
40% starch, 20% casein, 15% sucrose, 10% wheat bran, 5% oil for 27 d	Mice (*n* = 6)	80 vs. 2% amylose	(Wang et al., 2002)
40% starch, 25% sucrose, 25% casein, 5% oil for 21 d	Rat (*n* = 6)	68 vs. 26% amylose corn starch	(Kishida et al., 2001)
71% starch, 7% oil for 21 d	Rat (*n* = 8)	High amylose vs. raw potato vs. normal starch	(Lopez et al., 2001)
58.3% starch for 10-13 d	Rat (*n* = 8)	High amylose starch at 10 vs. 0% of diet	(Saito et al., 2001)
60-68% rice, 6% oil for 21 d	Pig (*n* = 8)	Brown vs. white rice	(Bird et al., 2000)
63% starch, 20% casein, 10% oil for 28 d	Rat (*n* = 7)	RS at 1.8 vs. 1.3% of diet	(Cheng and Lai, 2000)
35% starch, 20% fat for 30 d	Rat (*n* = 6)	SDS, RS respectively; 13.8, 65 vs. 17.8, 61.8 vs. 53.7, 16.5%	(Ferguson et al., 2000)
60 % starch for 5 d	Pig (*n* = 4)	Raw potato vs. high amylose corn starch	(Martin et al., 2000)
56-57% starch, 1 meal	Pig (*n* = 5)	22 vs. 71% 180-min in vitro digestibility	(Noah et al., 2000)
40 g starch, 1 meal	Human (*n* = 7)	62 vs. 26% amylose	(Vonk et al., 2000)
64% starch or sucrose, 16% casein, 12% oil, 2.5% cellulose for 9 months	Rat (*n* = 8)	Corn starch vs. sucrose	(Cresci et al., 1999)
89-93 g starch, 2.-1.8% fat, 1 meal	Human (*n* = 9)	16.5 vs. 1.3g RS (Approx. 50 vs. 64% in vitro digestibility)	(Hoebler et al., 1999)
60% corn starch, 18% casein, 14% tallow, 3.8% sucrose, 35 days	Rat (*n* = 8)	RS as 6 vs. 0% of diet	(De Schrijver et al., 1999a)
54% wheat and barley grain; 20% corn starch, 11% cassava, 6% oil, 35 d (rat) 11 d (pig)	Rat (*n* = 8) Pig (*n* = 8)	RS as 6 vs. 0% of diet	

Table 1.4 contd…

Diets and duration	Species (n)	Treatments	Reference
54% wheat and barley grain; 20% corn starch, 11% cassava, 6% oil, 35 d (rat) 11 d (pig)	Pig (*n* = 8) Rat (*n* = 8)	6 vs. 0% RS	(De Schrijver et al., 1999b)
46% sucrose or starch, 23% casein, 23% oil for 28 d	Rat (*n* = 8)	31% regular corn starch and 15% retrograded corn starch vs. sucrose	(Silvi et al., 1999)
58-61% carbohydrate, 15-16% fat for 14 d	Human (*n* = 24)	27.9 vs. 21.5. vs. 2.3g RS	(Jenkins et al., 1998)
50 g starch, 1 meal	Human (*n* = 8)	Retrograded vs. gelatinized starch	(Achour et al., 1997)
Commercially prepared food with starch + Bifidobacteria for 7d	Pig (*n* = 12)	High amylase starch vs. normal corn starch	(Brown et al., 1997)
60% starch, 18% casein, 12% oil, 3% cellulose for 5 months	Rat (*n* = 10)	10 vs. 0% of starch as RS	(Kleessen et al., 1997)
37.5% of energy as fat, 50% of energy as carbohydrate (of which 48.2% was as starch) and 12.5% of energy as protein for 21 d	Pig (*n* = 8)	96 vs. 56 vs. 0% of starch as high amylose corn starch	(Topping et al., 1997)
64 % pea and 63% corn starch, 9-10% oil for 6 d	Pig (*n* = 4)	Pea vs. corn starch	(van der Meulen et al., 1997a)
63 - 66% starch, 22-25% fat for 9 d	Pig (*n* = 4)	66% pea vs. 32+32% corn+pea vs. 63% corn starch	(van der Meulen et al., 1997b)
17.5 g starch for 3 d	Pig (*n* = 5)	1.93 vs. 0 g RS / 200 mL liquid diet	(Morais et al., 1996)
25% of carbohydrate in diet replaced by starch, fat < 50 g, 1 meal	Human (*n* = 29)	85 % amylose (33%RS) vs. Normal starch	(Noakes et al., 1996)
70% starch diet for 5 d	Rat (*n* = 7)	9.7 vs. 3.5 vs. 0.2 vs. 0 g RS/day	(Berggren et al., 1995)
51, 28, and 34% energy from carbohydrate, starch, fat respectively, for 98 d	Human (*n* = 24)	70 vs. 30% amylose	(Behall and Howe, 1995)
51.4% starch, 20% casein, 8.5% sucrose, 5% wheat bran, 5% oil for 63 d	Rat (*n* = 6)	Starch as 61 vs. 0% amylose	(Byrnes et al., 1995)
45-51.4 g starch, 5.2 g lard and oil for 42 d	Rat (*n* = 8)	RS as 14.3 vs. 0.1% of diet	(de Deckere et al., 1995)
RS diet for 28 d	Human (*n* = 24)	30 vs. 0 g RS per day	(de Roos et al., 1995)

Table 1.4 contd…

Diets and duration	Species (n)	Treatments	Reference
45 - 46.8% starch, 5.5 - 6.5 fat, 1 meal	Human (*n* = 9)	36 vs. 49 vs. 85% 180-min in vitro digestibility (29 vs. 23 vs. 7 g RS)	(Granfeldt et al., 1995)
40.4 g starch, 5.1 g lard for 21 d	Rat (*n* = 10)	RS as 14 vs. 1.2% of diet	(Verbeek et al., 1995)
46.5 - 40.7 g starch, 1 meal	Human (*n* = 10)	27.1 vs. 0g RS	(Raben et al., 1994)
41.6 g starch, 5.3 g fat, 21 d	Rat (*n* = 10)	9.6 vs. 0.8 g RS	(de Deckere et al., 1993)
70% starch diet for 7 d	Rat (*n* =12)	24.7 vs 3% RS	(Schulz et al., 1993)
15% or 2% energy as amylose in breakfast; 17% or 11% of energy as amylose in lunch	Human, (*n* = 22)	High vs. low amylose	(Weststrate and van Amelsvoort, 1993)
59 g starch, 15.5 g fat, 1 meal	Human (*n* = 10)	5 vs. 3.5 g RS	(Holm and Bjorck, 1992)
71% starch, 5% oil for 21 d	Rat (*n* =12)	60% corn and 11% wheat vs. 71% wheat starch	(Morand et al., 1992)
57% and 24 % energy from polysaccharides and fat, 1 meal	Human (*n* = 22)	45 vs. 0% amylose	(van Amelsvoort and Weststrate, 1992)
50 g available carbohydrate, 1 meal	Human (*n* = 11)	3 rice varieties with 27% amylose starch	(Panlasigui et al., 1991)
52 and 37% energy from carbohydrate and fat; 35% of diet as starch, 60% of carbohydrate calories from corn starch for 28 d	Human (*n* =12)	70 vs. 30% amylose	(Behall et al., 1989)
wheat and manihot starch, 1 meal	Human (*n* = 6)	71 vs. 58 vs. 16 vs. 2% 180-min in vitro digestibility	(Bornet et al., 1989)
1 g starch, 0.33 g fat per kg body wt., 1 meal	Human (*n* = 25)	70 vs. 30% amylose	(Behall et al., 1988)
55.6 g starch, 1 meal	Human (*n* = 33)	23-25 vs. 15-17 vs. 14-17 vs. 0% amylose	(Goddard et al., 1984)

1.4.4 Profile of metabolites

Starch chemistry can affect the profile of metabolites such as glucose, lactate, cholesterols, triglycerides, and short-chain fatty acids (SCFA) in the systemic circulation. Reduced episodes of hyperglycaemia and improved lipid responses such as lower cholesterol and triglycerides levels are beneficial for the management of diseases related to carbohydrate metabolism such as diabetes and obesity (Seal et al., 2003; Wolever, 2003; Ells et al., 2005). Lower and sustained availability of glucose in systemic circulation may also have implications for physical and mental performance (Benton et al., 2003), satiety (Leathwood and Pollet, 1988), and management of cardio-vascular diseases (Jenkins et al., 2002). Therefore starches with lower rates and extent of digestion may be useful in maintaining desired profile of metabolites for the management of diseases related to carbohydrate metabolism.

1.4.4.1 Glucose

Starch effects on postprandial glucose response such as peripheral or portal glucose levels or net portal glucose absorption have been extensively studied. Of ileal-digested starch, approximately 89% was absorbed as glucose in the portal circulation (Giusi-Perier et al., 1989; van der Meulen et al., 1997a). As a percentage of digested starch, glucose absorption was decreased in animals fed SDS compared to RDS (Morand et al., 1992; van der Meulen et al., 1997a). The findings suggest that either glucose is metabolized by the gut wall or a portion of starch is fermented in the small intestine after feeding SDS.

Meta-analysis of 25 studies with a total of 38 comparisons indicated that SDS reduced ($P < 0.001$) postprandial glucose response (**Figure 1.6**). In the analysis, 19 comparisons showed lower glucose response and 1 comparison showed greater glucose response of SDS compared to RDS; while 18 comparisons showed no effect of SDS on glucose response compared to RDS. In the study where SDS diet increased glucose response, the starch chemistry effect was probably confounded by a processing effect because the starches were gelatinized before feeding (van Amelsvoort and Weststrate, 1992). These studies indicate that starch containing as low as 20% greater amylose or 15.2 g more RS and 14% greater 180-min in vitro digestibility sometimes lowered postprandial glucose; while, starch containing up to 40% greater amylose, 24 g more RS and 49% greater 180-min in vitro digestibility did not result lower postprandial glucose. This finding clearly shows that the individual starch characteristics such as amylose, RS and 180-min in vitro digestibility alone may not explain starch effect on postprandial glucose responses.

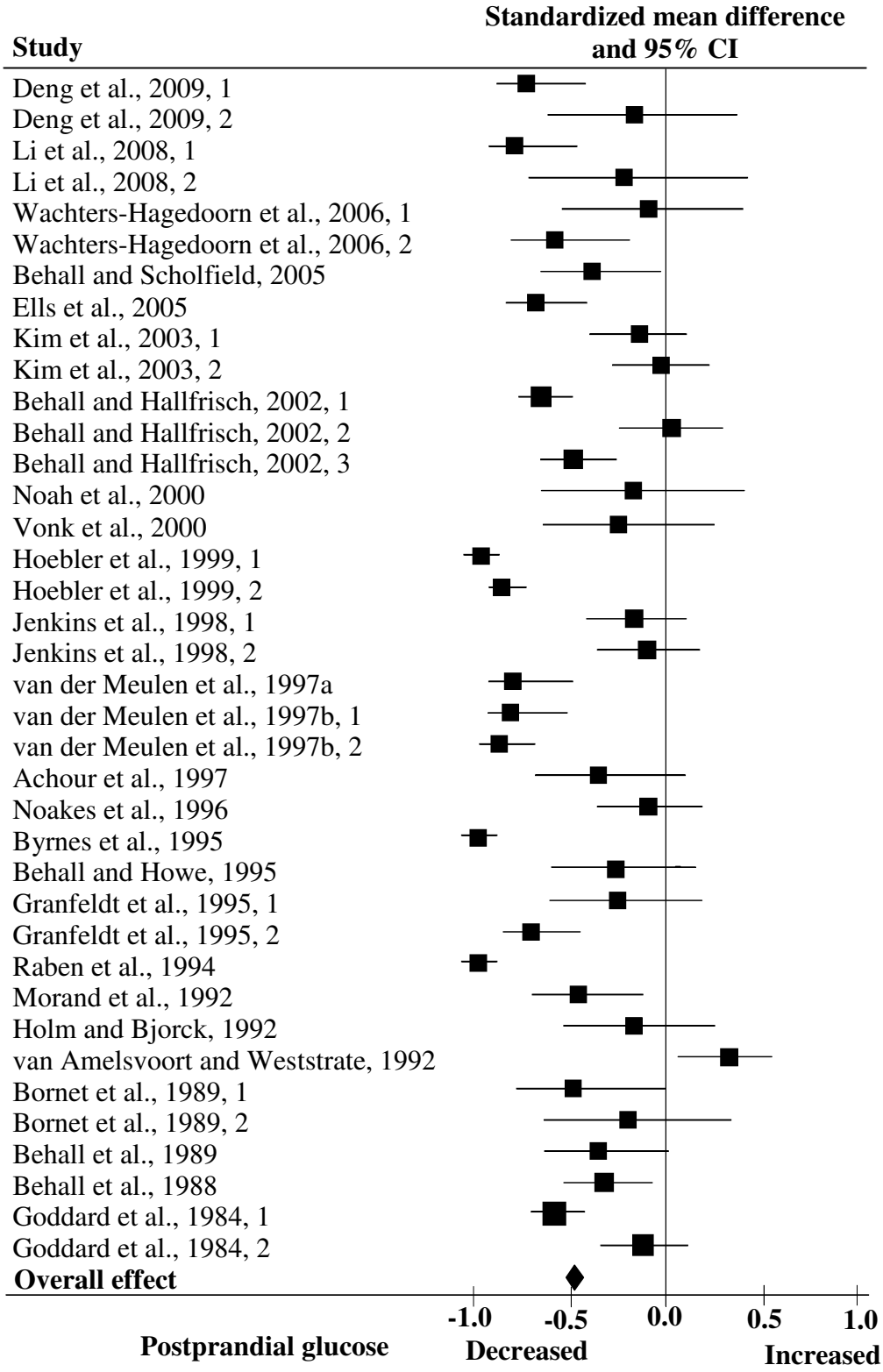

Figure 1.6 Meta-analysis of effect slow starch digestion on postprandial glucose level; CI, confidence interval; test for overall effect: $Z = -10.3$, $P < 0.001$.

1.4.4.2 Lactate

Lactate is an intermediate- and / or end-product of gut microbial fermentation and glucose and glutamine metabolism (Argenzio and Southworth, 1975; Giusi-Perier et al., 1989) in the gut epithelium. As starch is a major component of monogastric diets, glucose metabolism is a major source of lactate in portal circulation. Gut mucosa can metabolize up to 41% of absorbed glucose into lactate (Porteous and Pritchard, 1972). In pigs, 6.7 to 7.3% of total starch consumed was recovered as plasma lactate (Noah et al., 2000); hence, portal lactate and glucose were correlated positively (Giusi-Perier et al., 1989; van der Meulen et al., 1997a).

Starch chemistry can affect the plasma lactate profile. A meta-analysis of 5 studies with a total of 7 comparisons indicated that SDS reduces ($P < 0.001$) postprandial lactate response in monogastrics. In the analysis, SDS, compared to RDS, decreased lactate response in 3 comparisons and did not affect in 4 comparisons (**Figure 1.7**). The findings indicate that starch had to differ more than 18% in amylose content or have a 49% greater 180-min in vitro digestibility to lower postprandial lactate. In the study (van der Meulen et al., 1997a) where SDS numerically increased postprandial lactate response, SDS did not lower net portal lactate flux up to 6 h postprandial and maintained positive lactate flux as opposed to negative portal lactate flux in RDS after 6 h postprandial. Hence, lactate is net produced as long as glucose is being absorbed in the small intestine and the kinetics of postprandial plasma lactate may be different based on the rates and extent of enzymatic digestion of starch in the gut.

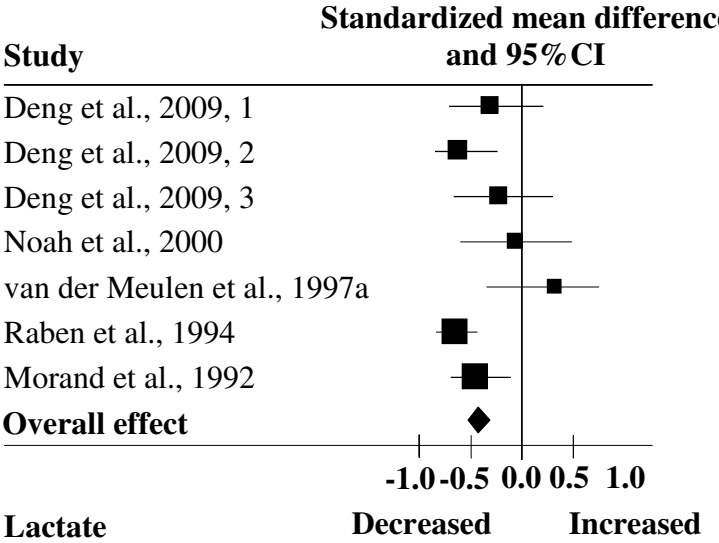

Figure 1.7 Meta-analysis of effect slowly digestible starch on postprandial lactate level; boxes and horizontal lines indicate individual study effects and confidence interval; the size of the box is proportional to the relative weight of the study; diamond indicates the overall effect; vertical line drawn at "zero" indicates no effect; CI, confidence interval; test for overall effect: Z = -3.94, *P* = 0.008.

1.4.4.3 Total SCFA and butyrate

Short-chain fatty acids are the principal end-products of fermentation in the gut, mainly in the large intestine (Argenzio and Southworth, 1975). Among SCFA, butyrate is mainly utilized by gut epithelial cells; propionate is metabolised in the liver (Cheng and Lai, 2000); and acetate primarily appears in the systemic circulation. The SCFA are useful preventative or therapeutic agents in various intestinal diseases (Scheppach et al., 2001; Bauer-Marinovic et al., 2006). SCFA mainly butyrate improves immune surveillance by increasing secretory IgA, T-cells, and luminal mucin (Roediger, 1990; Cummings and Englyst, 1991; Morita et al., 2004) and protects colonocytes from oxidative stress, and inflammatory reactions (Hamer et al., 2009). Among SCFA, butyrate increases growth and differentiation of the gut epithelium and improvement of immune-surveillance

(Roediger, 1990; Cummings and Englyst, 1991; Scheppach, 1994) and these properties may be useful in reducing the risk of infectious and degenerative enteric diseases. Furthermore, butyrate may reduce cancer risks by acting as a cancer-suppressing agent by inducing cell cycle arrest and differentiation and apoptosis of carcinoma cells (Archer et al., 1998; Brouns et al., 2002). Butyrate production in the colon could be more beneficial for a healthy colon, since uptake of butyrate by the colonocytes is much higher than small intestinal epithelium. Starch can be fermented to butyrate without any benefit to the colonic epithelium. In order to prevent or to treat diseases of the colon, it would appear to be necessary to select starch sources that will reach colon for microbial fermentation (Martin et al., 2000).

Starch chemistry can affect the production and absorption of microbial fermentation products in monogastrics. A meta-analysis of 24 studies with a total of 26 comparisons indicated that SDS increases ($P < 0.001$) concentration of total SCFA in the gut lumen and/or systemic circulation in monogastrics. Postprandial total SCFA profile was increased by SDS compared to RDS in 13 comparisons and was not affected by SDS compared to RDS in 13 comparisons (**Figure 1.8**). These studies indicate that starch containing as low as 10% greater amylose or 6% greater RS sometimes increased postprandial total SCFA profile; while, starch containing up to 78% greater amylose and 13.6 % greater RS did not increase postprandial total SCFA profile. This finding clearly shows that amylose and RS alone may not explain starch effects on postprandial total SCFA responses.

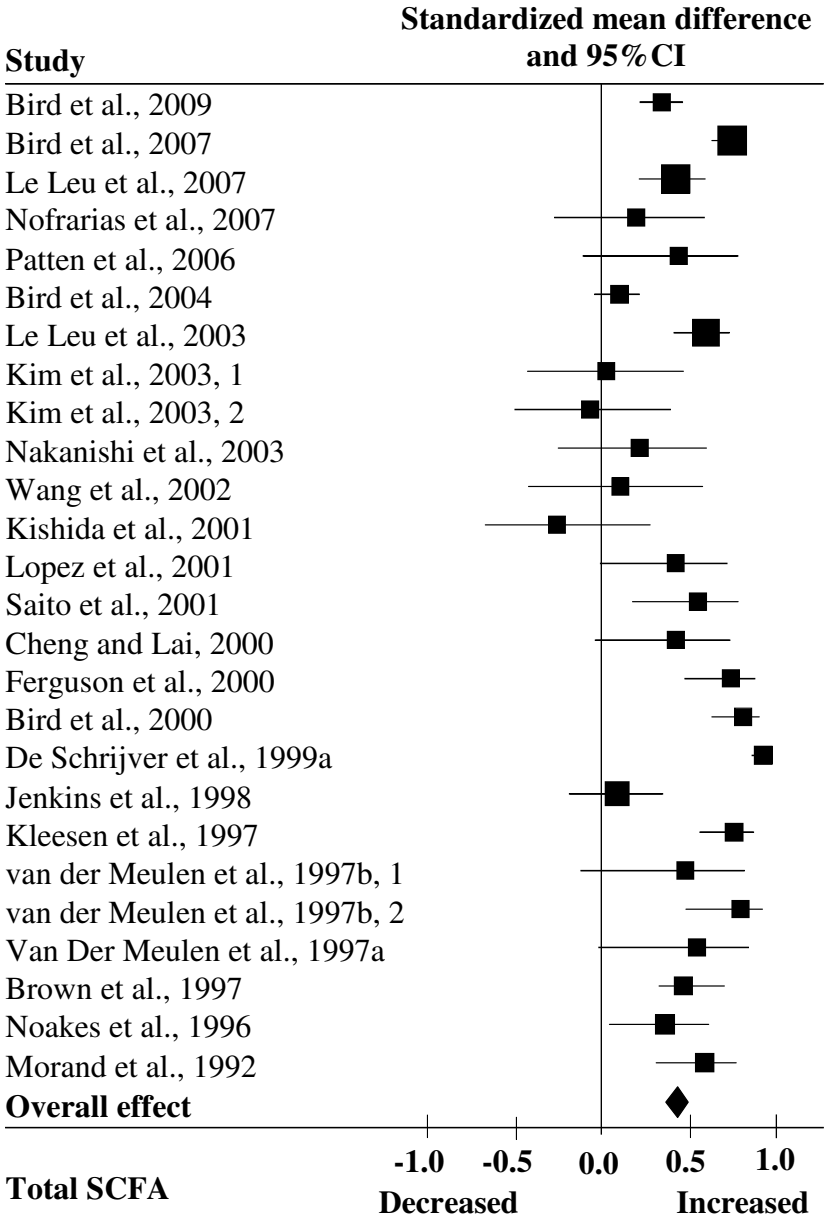

Figure 1.8 Meta-analysis of effect slowly digestible starch on postprandial total SCFA; boxes and horizontal lines indicate individual study effects and confidence interval; the size of the box is proportional to the relative weight of the study; diamond indicates the overall effect; vertical line drawn at "zero" indicates no effect; CI, confidence interval; test for overall effect: $Z = 9.46$, $P < 0.001$.

Similarly with regards to butyrate, a meta-analysis of 23 studies with a total of 24 comparisons indicated that SDS increases ($P < 0.001$) concentration of butyrate in the gut lumen and/or systemic circulation. In the analysis, SDS, compared to RDS, increased postprandial butyrate profile in 15 comparisons, decreased in 1 comparison (RS at 1.8 vs. 1.3% of diet); while, had no effect in 8 comparisons (**Figure 1.9**). These studies showed that starch containing as low as 55% greater amylose or 6.2% greater RS content sometimes increased postprandial butyrate profile; while, starch containing up to 45% greater amylose and 48.5% greater RS did not result greater postprandial butyrate profile. This finding clearly shows that the individual starch characteristics such as amylose and RS alone may not explain starch effects on postprandial butyrate responses.

As higher SCFA content decreases the pH in the gut lumen, previous studies consistently showed that SDS decreases ileal (Bird et al., 2000; Bird et al., 2007), cecal (de Deckere et al., 1995; Silvi et al., 1999; Lopez et al., 2001; Le Leu et al., 2002; Morita et al., 2004; Bird et al., 2007), colon; (Bird et al., 2000; Bird et al., 2007) and fecal pH (Verbeek et al., 1995; Bird et al., 2000; Le Leu et al., 2002; Bird et al., 2007).

In addition to SCFA, CO_2, H_2 and CH_4 gases are produced during microbial fermentation in the gut. The formation of H_2 and CH_4 is unique to anaerobic bacteria, because cells of higher order species do not produce these gases. Hence, the breath level of both gases is correlated with the degree of colonic fermentation

(Le Marchand et al., 1992). The SDS increased H_2 and CH_4 excretion compared with RDS (Achour et al., 1997; Jenkins et al., 1998) indicating greater anaerobic fermentation in colon for SDS compared to RDS.

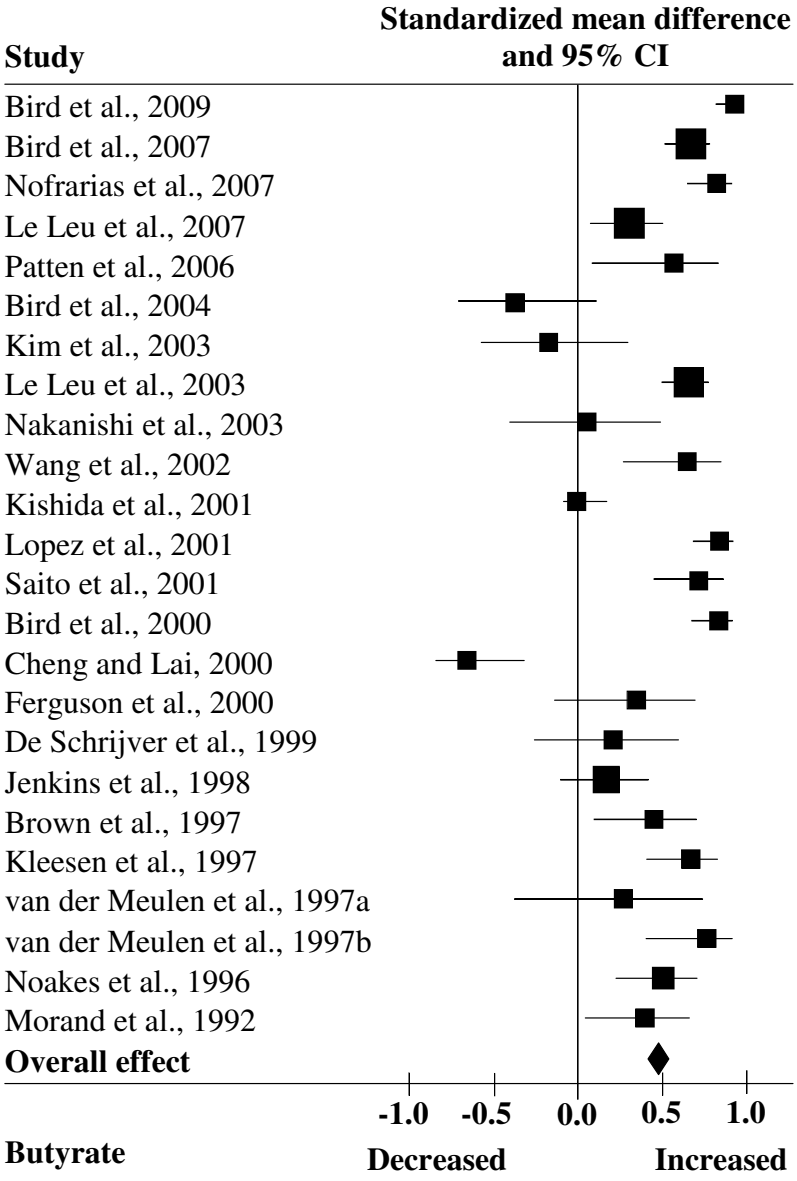

Figure 1.9 Meta-analysis of effect of slowly digestible starch on postprandial butyrate; boxes and horizontal lines indicate individual study effects and confidence interval; the size of the box is proportional to the relative weight of the study; diamond indicates the overall effect; vertical line drawn at "zero" indicates no effect; CI, confidence interval; test for overall effect: $Z = 9.36$, $P < 0.001$.

The SDS works as a fermentable fiber and greater proportion of the starch is utilized for SCFA production in the expense of glucose. As energy production from SCFA is at least 14% lower than that from glucose (Livesey, 1992; Jorgensen et al., 1997) mainly due to lower ATP yield per mole basis, SDS is energetically less efficient than RDS. The lower energy efficiency might be the reason for lower feed efficiency and growth rate in animals fed SDS compared to RDS.

1.4.4.4 Metabolites of protein and amino acid digestion

Starch chemistry can affect digestion, absorption and metabolism of protein and amino acids. Slowly digestible starch increases amino acid utilization by enterocytes (Li et al., 2008), and thus, may reduce amino acid appearance in portal circulation. The net absorption of glutamine, a major energy source for enterocytes (Wu et al., 1995), can be negligible or negative while feeding SDS (van der Meulen et al., 1997a) indicating glutamine uptake by the intestinal epithelium from systemic circulation. However, a more gradual supply of glucose to the intestinal tissues can result 20% higher amino acid absorption (Giusi-Perier et al., 1989). Previously, moderately rapidly digestible starch had greater 8 h net portal uptake of arginine, leucine, methionine, threonine, glutamate, and tyrosine compared to both RDS and SDS diets (Li et al., 2008).

Ammonia is produced by the intestinal microflora and the intestinal epithelium from amino acids such as glutamine (Rerat and Buraczewska, 1986; Remesy and

Demigne, 1989). Ammonia is used for *de novo* synthesis of bacterial protein in the gut, and thus, SDS can reduce portal absorption of ammonia (van der Meulen et al., 1997a). Bacteria may grow with ammonia as the sole source of nitrogen, and some bacteria even prefer ammonia to amino acids (Wrong and Vince, 1984; Mosenthin et al., 1992). In addition, net transfer of urea from circulation to the gastrointestinal tract occurs during high dietary starch intake (Rerat and Buraczewska, 1986; van der Meulen et al., 1997b), and such transfer is higher with SDS (van der Meulen et al., 1997a). Other products of protein metabolism, such as creatinine (Jenkins et al., 1998), blood urea nitrogen and uric acid (Behall et al., 1989) were not affected by starch chemistry.

1.4.4.5 Profile of fat metabolism indicators

Starch chemistry can affect fat metabolism indicators such as cholesterols, lipoproteins, triglycerides and bile acids. Blood cholesterols mainly higher level of low density lipoproteins (LDL) and lower level of high density lipoproteins (HDL) and blood lipids are considered as risk factors for cardiovascular diseases in human (Assmann et al., 1991; Lewis, 1998). In animals, these variables may influence lean vs. fat deposition, thus, meat quality. Slowly digestible starch increases respiratory quotient and fat oxidation (Higgins et al., 2004) and thus decrease body fat content as indicated by decreased fat pads (de Deckere et al., 1993; de Deckere et al., 1995; Kishida et al., 2001; Kim et al., 2003).

Meta-analysis of 9 studies indicated that SDS decreased ($P < 0.001$) total cholesterol level in peripheral plasma. Slowly digestible starch, compared to RDS, lowered total cholesterol level in 5 comparisons and had no effect in 4 comparisons (**Figure 1.10**). These studies showed that starch containing as low as 8.8 g more RS content or 40% greater amylose sometimes decreased total cholesterol; while, starch containing up to 60% greater amylose and 14.2% greater RS did not lower total cholesterol in peripheral plasma. This finding indicates that starch effect on total cholesterol in peripheral plasma may not be explained by starch variables such as amylose and RS content.

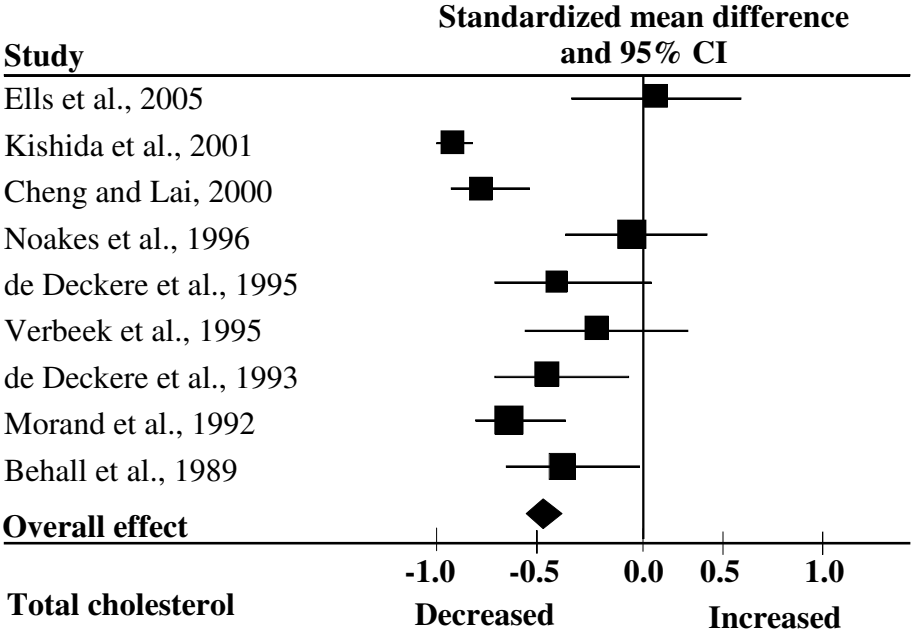

Figure 1.10 Meta-analysis of effect of slowly digestible starch on total blood cholesterol; boxes and horizontal lines indicate individual study effects and confidence interval; the size of the box is proportional to the relative weight of the study; diamond indicates the overall effect; vertical line drawn at "zero" indicates no effect; CI, confidence interval; test for overall effect: $Z = -7.23$, $P < 0.001$.

In addition, fecal excretion of total cholesterol was increased by SDS (Lopez et al., 2001). Both HDL and LDL were either decreased (Cheng and Lai, 2000; Kishida et al., 2001) or not affected by SDS (Behall et al., 1989; Noakes et al., 1996; Ells et al., 2005). The conversion of cholesterol into bile acids and sterols and their subsequent fecal excretion is a major pathway for elimination of cholesterol from the body (Turley and Dietschy, 1988). Effect of starch chemistry on fecal excretion of bile acid and neutral sterols was inconsistent. Fecal excretion of bile acid was either increased (Verbeek et al., 1995; Kishida et al., 2001) or decreased (Cheng and Lai, 2000) by SDS compared to RDS. Similarly, fecal excretion of neutral sterols was either increased (Verbeek et al., 1995; Lopez et al., 2001) or not affected (Kishida et al., 2001) by SDS compared to RDS.

Triglycerides are essential for normal body metabolism; however, excess circulating triglycerides (Labreuche et al., 2009) and free fatty acids (Jouven et al., 2001) are risk factors for cardiovascular diseases and obesity. A meta-analysis of 8 studies indicated that SDS decreases ($P < 0.001$) triglycerides level in circulation. In the analysis, SDS, compared to RDS, decreased triglyceride level in 5 comparisons, and had no effect in 3 comparisons (**Figure 1.11**). These studies indicated that starch containing as low as 8.8 g more RS content or 40% greater amylose sometimes decreased triglycerides; while, starch containing up to 60% greater amylose did not affect triglycerides. This finding indicates that starch effect on triglycerides profile in peripheral plasma may not be reliably explained by starch characteristics such as amylose and RS content.

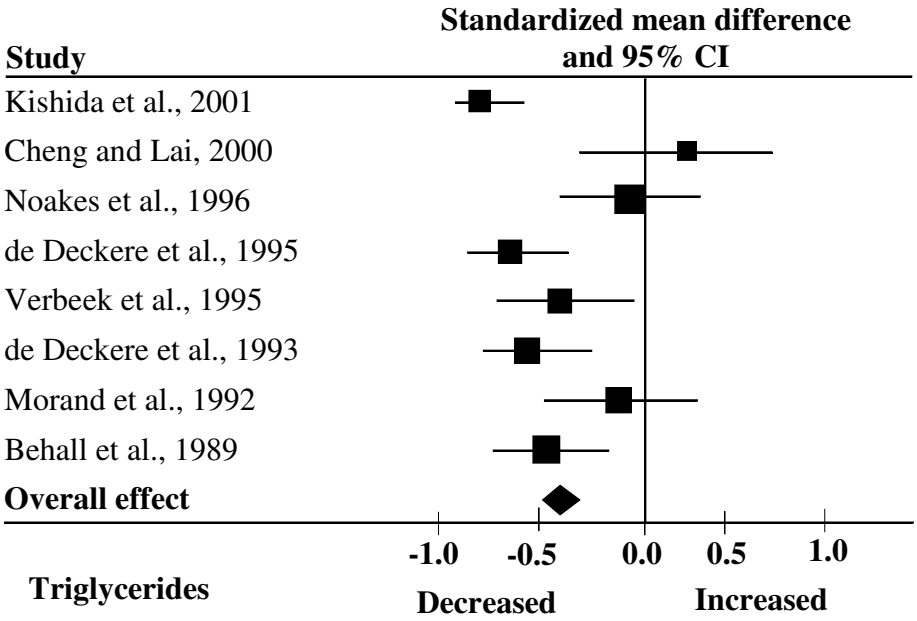

Figure 1.11 Meta-analysis of effect of slowly digestible starch on blood triglycerides; boxes and horizontal lines indicate individual study effects and confidence interval; the size of the box is proportional to the relative weight of the study; diamond indicates the overall effect; vertical line drawn at "zero" indicates no effect; CI, confidence interval; test for overall effect: Z = -6.02, $P < 0.001$.

Free fatty acids are either decreased (Morand et al., 1992; van Amelsvoort and Weststrate, 1992; Weststrate and van Amelsvoort, 1993) or not affected (Higgins et al., 2004; Ells et al., 2005) by SDS compared to RDS. Serum lipids and lipoprotein (Jenkins et al., 1998) and liver fatty acid synthesis (de Deckere et al., 1993) were not affected by SDS.

1.4.4.6 Gut mineral availability and absorption

Starch chemistry can affect utilization of minerals such as Ca, Mg, P, Zn, Fe, and Cu in the body. Starch chemistry modulates mineral solubility and thus, can influence their availability in the gut and absorption in circulation. In addition to minerals' essential role in several physiological processes in the body, minerals

beneficially affect gut environment. For instance, Ca availability in the large intestine may exert a protective effect on the colon epithelium (Wargovich et al., 1983) and inhibit the cytotoxicity of potential carcinogens, such as bile acids or fatty acids (Govers and Van der Meet, 1993). Starch types may not have any effect on strictly regulated Ca absorption in the small intestine (Bronner et al. 1986); but, SDS could favour Ca absorption in the large intestine in several ways: hypertrophy of the cecal wall and greater surface area, increase in soluble Ca, and accelerated blood flow due to microbial fermentation (Schulz et al., 1993; Younes et al., 1996). Although, SDS did not consistently increased Ca absorption from the gut, the starch increased the retention of Ca in the body (Schulz et al., 1993; Morais et al., 1996; Kishida et al., 2001). In addition, effect of SDS on the concentration of Ca in the large intestine is inconsistent; either increased (Schulz et al., 1993; Younes et al., 1996) or decreased (Bird et al., 2000).

Large intestine is a major site of Mg absorption, which is consistent with the fact that Mg in foods from plant source becomes fully available after the microbial digestion of carbohydrate (Hardwick et al., 1990). Slowly digestible starch increases Mg solubility in the cecum, its absorption (Schulz et al., 1993), and retention of Mg in the body (Lopez et al., 2001). Similarly, P solubility was greater in the cecal content (Schulz et al., 1993) but P absorption was not increased by SDS compared to RDS (Morais et al., 1996; Kishida et al., 2001). In addition retention of Ca, Mg, Zn, Fe, and Cu was increased by SDS compared to RDS (Lopez et al., 2001). And the absorption of Fe is increased and that of Zn

was not affected (Younes et al., 1996) by SDS (Morais et al., 1996; Kishida et al., 2001). Therefore, SDS may improve mineral absorption via increasing solubility of minerals in the large intestine.

1.4.5 Endocrine responses

As starch chemistry can modulate digestion and absorption of nutrients, it may influence hormonal responses that are directly and indirectly linked to the altered digestion, absorption and metabolism of nutrients. Endocrine responses such as insulin, glucagon, glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), and thyroid hormones and metabolic responses such as lipogenesis, glycolysis and protein deposition correspond to the postprandial glycemia and can differ in SDS and RDS.

1.4.5.1 Insulin

Insulin is responsible for uptake and storage of glucose by cells in the liver, muscle and adipose tissues. Intestinal starch digestion affects blood glucose profile and chronic hyperglycemia results insulin resistance ultimately leading to type 2 diabetes. Effect of starch types on postprandial insulin has been extensively studied. A meta-analysis of 21 studies with a total of 28 comparisons indicated that SDS decreases ($P < 0.001$) peripheral insulin. Slowly digestible starch,

compared to RDS, lowered postprandial insulin level in 15 comparisons and had no effect in 13 comparisons (**Figure 1.12**). Starch containing as low as 15.2 g more RS content or 24% greater amylose and 14% greater in vitro digestibility sometimes decreased insulin; while, starch containing up to 40% greater amylose, 16 g greater RS and 49% greater in vitro starch digestibility did not lower insulin.

The inconsistency of SDS effects on peripheral insulin indicates that starch effects on plasma insulin may not be explained by starch variables such as amylose and RS content. Or perhaps peripheral insulin is not a reliable indicator of insulin secretion. Instead peripheral C-peptide level may be used to explain insulin secretion because C-peptide is released in equimolar amounts from proinsulin in the pancreas and has a lower hepatic extraction and renal excretion than insulin (Polonsky and Rubenstein, 1984); thus C-peptide is a more reliable indicator of insulin release in peripheral circulation.

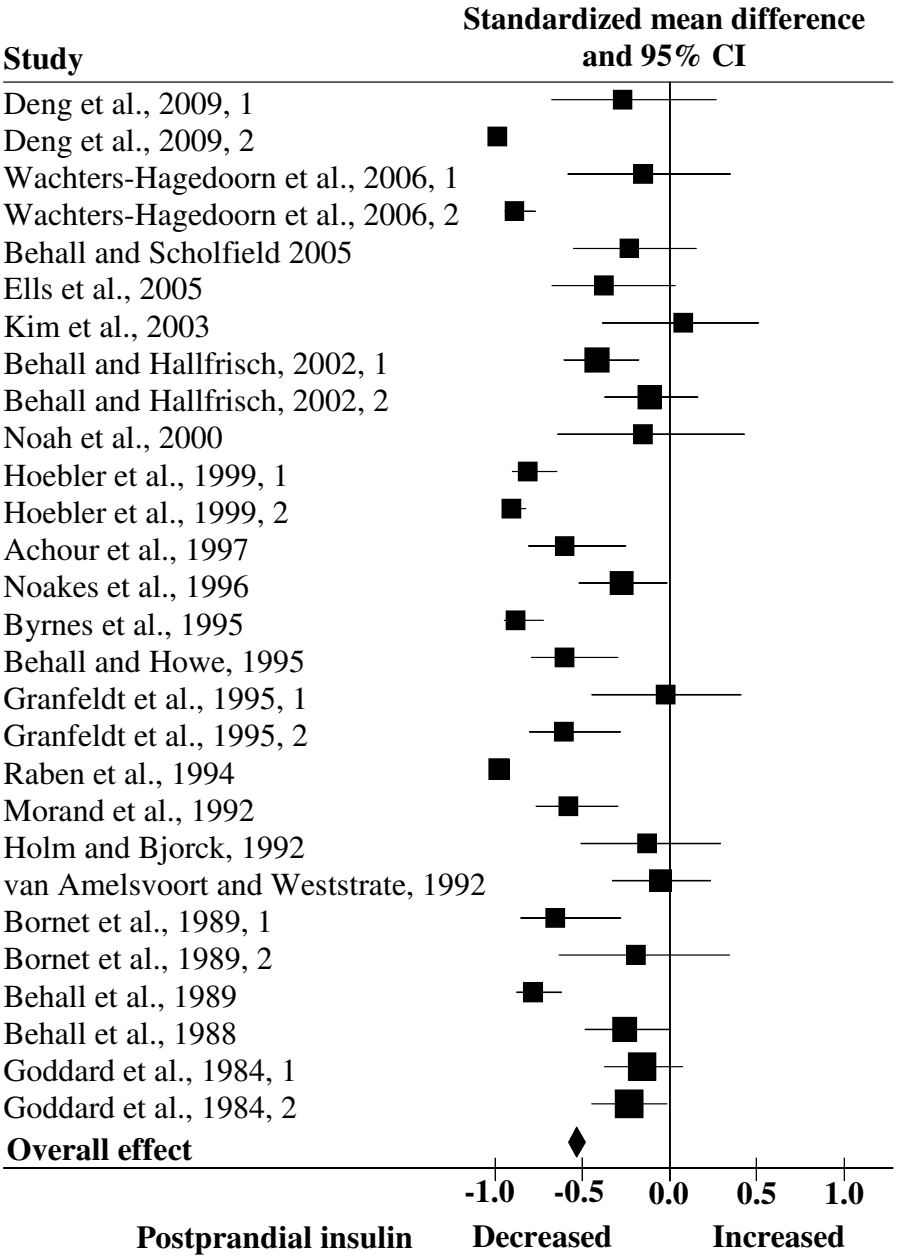

Figure 1.12 Meta-analysis of effect slowly digestible starch on postprandial insulin level; boxes and horizontal lines indicate individual study effects and confidence interval; the size of the box is proportional to the relative weight of the study; diamond indicates the overall effect; vertical line drawn at "zero" indicates no effect; CI, confidence interval; test for overall effect: Z = -9.2, $P < 0.001$.

1.4.5.2 Incretins

Incretins play an important role in the regulation of blood glucose via amplification of glucose-induced insulin secretion (Kellett et al., 2008). Incretin-stimulated insulin secretion accounts for at least 50% of the total insulin secreted after oral glucose consumption (Kim and Egan, 2008). GIP is released from K cells present in the duodenal and proximal jejunal mucosa, whereas GLP-1 is released from L cells located mainly in the mucosa of distal jejunum, ileum and colon (Nauck, 2009). Nutrients such as glucose in the lumen stimulate signal transduction via the apical membrane of K and L cells that facilitates incretin secretion (Raybould, 2007). Slowly digestible starches decrease incretins (both GIP and GLP-1) secretion from 0 to 2 h postprandial but increases from 2 to 4 h postprandial (Wachters-Hagedoorn et al., 2006). Compared with fasting values, GIP increased by a factor of 10 after meal containing RDS; while, no increase was observed after meal containing SDS (Raben et al., 1994). Total GLP-1 secretion, however, was not affected by starch types (Raben et al., 1994).

1.4.5.3 Other hormones

In addition to insulin and incretins, starch chemistry may influence other endocrine responses such as thyroid hormone and glucagon. Plasma T3 of piglets fed diet with SDS was lower indicating lower metabolic rate of piglets fed SDS than RDS. This reduced metabolism is also reflected in low plasma glucose and lactic acid levels (Deng et al., 20010). With regard to starch chemistry effects on glucagon, slow digestible starch did not have consistent effects on postprandial

glucagon level in circulation (Behall et al., 1989; Behall and Hallfrisch, 2002; Behall and Scholfield, 2005; Behall et al., 2006). SDS had no effect on norepinephrine but decreased epinephrine level (Raben et al., 1994).

1.4.6 Gut microbial profile

Beneficial gut microflora such as bifidobacteria and lactobacilli establish an efficient barrier to the invasion and colonization of the gut by pathogenic bacteria and produces a range of metabolic substrates such as SCFAs, which are used by the host and stimulate the immune system in a non-inflammatory manner (Gibson and Roberfroid, 1995). In addition, SDS increases production of secretory IgA (Morita et al., 2004) and luminal mucin and protein synthesis in the gut (Deng et al., 20010).

The majority of gut bacteria are saccharolytic, and several bacterial groups, including *Eubacterium*, *Bacteroides*, and bifidodobacteria can ferment starch (Wang and Gibson, 1993; Brown et al., 1998). *Clostridium butyricu*m and bifidobacteria in particular are effective starch fermenters (Brown et al., 1998). These bacteria ferment starch primarily to acetate, propioinate, and butyrate. Bifidobacteria possess α-amylase and pullulanase activity required specifically for starch fermentation in the distal gut (Ryan et al., 2006). Although specific butyrate producing phylogenic cluster (/s) of bacteria have not yet been identified (Louis et al., 2007), numerous species belonging to *Clostridium* and *Eubacterium*

can ferment starch to produce butyrate (Barcenilla et al., 2000; Sharp and Macfarlane, 2000).

Starch chemistry affects enzymatic digestion of starch and thereby starch availability for microbial fermentation in the gut. Slowly digestible starch increases the digesta mass in the distal gut (**Figure 1.4**) which serves as a substrate for growth and multiplication of gut bacteria (Topping et al., 1997; Wang et al., 2002). In addition, increased digesta mass from SDS may protect the gut bacteria against bile acids, free fatty acids, partial glycerides, and other products of digestion that have bactericidal actions (Topping et al., 1997).

Meta-analysis of 10 studies indicated that SDS increases bifidobacteria population. Slowly digestible starch compared to RDS, increased ($P < 0.001$) bifidobacteria population in 9 studies and did not affect bifidobacteria population in 1 study (10 vs. 0% of diet as high amylose starch; **Figure 1.13**). These studies showed that starch containing as low as starch containing 6 g more RS increased bifidobatcteria population. Previously, both high amylose and waxy starch stimulated the growth of bifidobacteria in the large intestine indicating starch flow into the distal gut is more important than starch chemistry per se (Wang et al., 2002). The reason for this bifidogenic property of starch may be the presence of α-amylase and pullulanase activity, which is required for specific use of starch, in several strains of bifidobacteria (Ryan et al., 2006).

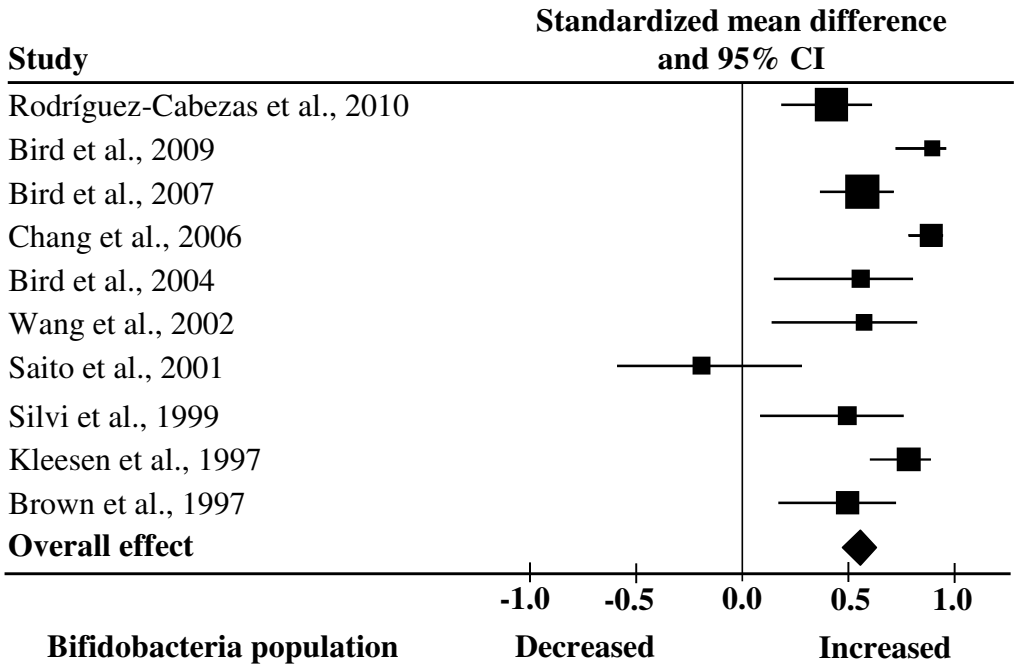

Figure 1.13 Meta-analysis of effect slowly digestible starch on bifidobacteria content in the feces and large intestinal content; boxes and horizontal lines indicate individual study effects and confidence interval; the size of the box is proportional to the relative weight of the study; diamond indicates the overall effect; vertical line drawn at "zero" indicates no effect; CI, confidence interval; test for overall effect: $Z = 10.87$, $P < 0.001$.

In addition to bifdobacteria, lactobacilli population increased after feeding starches (Brown et al., 1997; Kleessen et al., 1997; Wang et al., 2002; Morita et al., 2004; Bird et al., 2007) with sufficiently wide variation in RS (6 g in rats) or amylose content (40% in pigs). Starch chemistry effects on lactobacilli population may not be apparent when starch variability is less wide (2 vs. 0 g RS in rats (Rodríguez-Cabezas et al., 2010). Unlike inulin and fructo-oligosaccharides, which are used as prebiotics, SDS selectively increases the population of bifidobacteria and lactobacilli without increasing the population of total aerobes and coliforms *Escherichia coli* in feces and digesta (Brown et al., 1997; Morita et al., 2004; Bird et al., 2007).

1.4.7 Reasons for apparent inconsistencies on starch effects on physiological responses

Slowly digestible starch may reduce feed efficiency and profile of glucose, lactate, triglycerides, cholesterols and insulin profile and increases fecal bulk, SCFA profile and population of bifidobacteria and lactobacilli. However, these effects are not consistent among studies, as indicated by the meta-analyses in this review. The inconsistent response can be probably due to issues in following four areas: a) starch characterization; b) experimental design of treatments, c) response variables that were measured; and d) likely, errors in experimental procedures.

First, in most of the previous studies, starch characteristics such as amylose, RS, source of starch has been used to describe starch response in vivo. These characteristics have poor biological relevance and cannot properly differentiate foods with different rates of starch digestion. Starch characteristics that can reliably explain in vivo responses should be used to describe in vivo starch effects. Instead of amylose, RS or starch source, the rate and extent of in vitro starch digestibility at least up to 4 h should be consistently used to describe starch effects on in vivo responses. Second, experimental design of treatments is inconsistent. For instance, in few studies, the in vivo response observed were not sole effects of starch chemistry, the response were rather confounded by starch-associated compounds such as protein, fat, and fiber present in grains or diets. Similarly, inadequate description of diet has also contributed to the inconsistencies. Studies with similar difference in starch chemistry may not result

similar in vivo response if the amount of starches used in diets are different. Third, many of the previous studies have not measure accurate responses. For instance, to determine nutrient absorption, metabolite production, hormone secretion or bacterial growth, quantitative value (weight/time) needs to be measured as opposed to concentration (weight/volume). Because concentration is determined by the balance in the amount of certain compound entering and leaving the system, it may not necessarily indicate the total amount. Similarly, in vivo starch postprandial response needs to be observed at least up to 8 h postprandial (McCleary et al., 2002) as opposed to 2 h as has been the case of many studies on glucose and insulin profile. Fourth, it is important to minimize errors in experimental procedures to have reliable and repeatable in vivo response of treatments including starches.

1.5 Conclusion

Commonly used starch characteristics such as content of amylose and SDS and RS have poor biological relevance and may not be a valid indicator of in vivo starch responses. The meta-analyses showed that SDS decreased profile of glucose, lactate, insulin, triglycerides and total cholesterol in circulation and body weight gain and increases the production of butyrate and total SCFA, population of bifidobacteria and lactobacilli in the gut and fecal bulk. However, the difference in starch characteristics required to bring metabolically relevant changes is inconsistent among starches probably due to inadequate and improper starch characterization, treatment design and response measurement. Perhaps a

more in-depth characterization of starch and inclusion of starches covering a wide range in chemistry should be included in future studies to determine minimum level of starch chemistry variables in diet required to bring favourable physiological changes in monogastrics.

1.6 Overall hypotheses and objectives

The hypotheses of the thesis were a) in vitro starch digestion reflects the kinetics of in vivo glucose absorption and b) starch types with different physicochemical properties such as amylose content and in vitro rate of digestion affects nutrient digestion and fermentation, kinetics of nutrient absorption and endocrine response in pigs. Surgical models such as porto-arterial catheterized pigs and ileal-cannulated pigs were used to test the hypotheses.

The overall objective of the thesis was to determine the role of starch differing in physicochemical properties on nutrient digestibility and absorption, endocrine responses, and gut microbial profiles using swine model. The specific objectives were:

1. To determine the postprandial glucose response in portal vein-catheterized pigs fed diets containing purified starches with a wide range in the in vitro digestion kinetics and to describe the relationship between these two (Chapter 2).
2. To determine the role of starch chemistry on the kinetics of starch-derived nutrient absorption and insulin and incretins secretion and the

interrelationship among nutrients absorption and insulin and incretin secretion (Chapter 3).

3. To determine the effect of starch with different rate of in vitro digestibility on nuclear magnetic resonance (NMR) measured plasma metabolites in pigs (Chapter 4).
4. To evaluate the role of starch chemistry on intestinal nutrient flow, ileal and fecal fermentation characteristics and bacterial profile in pigs (Chapter 5).
5. To understand the effects of starch chemistry on ileal flow and apparent retention of minerals and the relation between fecal SCFA, fecal numbers of bacterial groups and post-ileal mineral and nitrogen flux in pigs fed starch diets with different rates of in vitro digestion (Chapter 6).

1.7 References

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Chapter 2. In vitro starch digestion kinetics, corrected for estimated gastric emptying, predicts portal glucose appearance in pigs[1]

2.1 Introduction

For nutritional purpose, starch types are commonly described by 3 fractions based on the rate and extent of in vitro enzymatic digestion: a) rapidly digestible starch (RDS) that acutely increases blood glucose, b) slowly digestible starch (SDS) that results in a drawn-out increase in blood glucose, and c) resistant starch (RS) that resists digestion by mammalian enzymes and thus does not yield glucose (Englyst et al., 1992; Englyst et al., 1999). The in vitro Englyst-assay (Englyst et al., 2000) determines these fractions RDS, SDS, and RS as digested within 20 min, 20 to 120 min, and not digested within 120 min, respectively (Englyst et al., 1992; Englyst et al., 1999), has a good repeatability, and is thus widely used for predicting in vivo glycemic responses.

The in vitro-based fractions RDS and SDS are better related with the glycemic index (R^2 = 0.62), which ranks carbohydrate-containing foods according to their in vivo glucose response (Wolever et al., 1991), than with actual carbohydrate components such as glucose, starch, sucrose, and fructose (R^2 = 0.17) (Englyst et al., 1999; Englyst et al., 2003). In addition, several studies (O'Dea K. et al., 1981; Muir and O'Dea, 1993; Englyst et al., 1996b; Weurding et al., 2001; Garsetti et

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P. R. Regmi, T. A. T. G. van Kempen, and R. T. Zijlstra designed research; P. R. Regmi and J. J. Matte conducted research; P. R. Regmi and T. A. T. G. van Kempen analyzed data; P. R. Regmi, T. A. T. G. van Kempen, and R. T. Zijlstra wrote the paper.

al., 2005), though not always consistent (Kim et al., 2003; Priebe et al., 2008), have indicated that a higher SDS and RS content in diets will reduce the rate and extent of in vivo starch digestion and, thus, maintains sustained and lower postprandial glucose responses in peripheral circulation. Therefore, starches ranging in SDS and RS content are used in many food preparations (Grabitske and Slavin, 2008) for the management of diseases related to carbohydrate metabolism (Behall et al., 2006; Grabitske and Slavin, 2008). Their actual contribution for maintaining the rate and extent of in vivo glucose absorption, however, is not clearly understood because accurate extrapolation from in vitro to in vivo is not yet possible. Furthermore, whether in vitro RS is an accurate predictor of starch resistant to enzymatic digestion in the small intestine is controversial, because starch digestion may continue well beyond 120 min of in vitro incubation (Muir and O'Dea, 1993).

The nutrient absorption kinetics in healthy human subjects is difficult to measure for ethical and technical reasons. Hence, pigs, having similar digestive anatomy and physiology (Pond and Lei, 2001) and a similar profile of nutrients and hormones in blood circulation (Spurlock and Gabler, 2008), can be a good model for understanding the kinetics of portal glucose appearance in humans. Therefore, a study was designed to determine the postprandial glucose response in portal vein-catheterized pigs fed diets containing purified starches with a wide range in the in vitro digestion kinetics and to describe the relationship between these two.

The hypothesis was that in vitro glucose release reflects the kinetics of in vivo glucose absorption.

2.2 Methods

2.2.1 In vitro digestion method validation and modification.

A 2 h in vitro technique described by Englyst et al. (2000) simulating gastric and small intestinal digestion is commonly used for determining in vitro digestibility of starch and for predicting the glycemic index of starchy foods (Germaine et al., 2008). In the present study the Englyst et al. (2000) technique was used with two modifications. First, we observed that the difference between glucose content measured 1 day after vs. immediately after in vitro enzymatic digestion was greater ($P < 0.001$) when 66% vs. absolute ethanol was used to stop the incubation (0.7 ± 0.23 vs. 0.0 ± 0.01 mmol/L, respectively). This difference indicated that 66% ethanol as used by Englyst et al. (2000) did not stop the enzymatic activity and thus, the time of glucose measurement relative to when the assay was stopped affected the readings. Absolute ethanol, on the other hand, completely stopped the enzymatic activity after incubation; therefore, absolute ethanol was used in the present study. Second, the time points at which subsamples for glucose analysis were taken were changed and the assay was extended to 8 h. Specifically, the 20 minute sampling time was moved forward to better characterize the sigmoidal digestion; 15 minutes was used as a compromise between what is practically feasible and what is desirable. The reason for extending the assay to 8 h was to not only match the in vivo time frame, but also

to ensure that even slowly digestible starches were digested to at least 95% of the plateau value. Going beyond 8 h, as done by, e.g., McCleary et al. (2002), was not deemed to add value and may result in an increasingly unstable assay due to, e.g., microbial contamination. Thus, samples were taken at 0, 15, 30, 60, 120, 240, 360, and 480 min to properly characterize starch digestion.

In the modified technique, 1 g ground sample (1 mm screen, Retsch grinder, model ZM1, Brinkman Instruments, Rexdale, Ontario, Canada) was added into a 50-mL tube. Samples were incubated in 10 mL pepsin solution, containing 0.05 g pepsin (P-7000; Sigma–Aldrich, Bornem, Belgium) and 0.05 g guar gum in 0.05 mol/L HCl for 30 min to mimic gastric digestion. For mimicking small intestine digestion, 10 mL of 0.25 mol/L sodium acetate ($C_2H_3NaO_2$) solution and 5 mL of an enzyme mixture containing 0.7 g pancreatin before centrifugation (P-7545; Sigma-Aldrich), 0.05 ml amyloglucosidase (EC 3.2.1.3; 61-002; 200 EU/mL; Englyst Carbohydrate Service Ltd., Southampton, Hampshire, UK), and 3 mg invertase (P-57629; Sigma-Aldrich) in water were added to the digestion solution and further incubated for up to 480 min. Incubations were carried out at 39°C under horizontal agitation; glass beads were added to enhance the efficacy of agitation and to provide a grinding action. At each sampling, an aliquot of 0.5 ml was taken to which absolute ethanol was added for stopping the digestion of the starch. Glucose content was determined in this blend using a glucose oxidase kit (Megazyme, Bray, Ireland).

2.2.2 Starch sources

Remyline AX-DR rice starch (Remy Industries, Leuven-Wijgmaal, Belgium), Remy B7 rice starch (Remy Industries), Nastar pea starch (Cosucra Group Warcoing, Warcoing, Belgium), and Gelose 80 corn starch (Penford Food Ingredients, Centennial, CO) samples with an expected wide range in digestibility kinetics were used in the study. The starches were considered rapidly (S1), moderately rapid (S2), moderately slow (S3), and slowly digestible (S4) starches based on their in vitro kinetics (maximal in vitro rate of starch digestion, %/min; 1.06, 0.73, 0.38, and 0.22). The starch sources had a wide range in content of SDS (starch digested from 20 to 120 min in vitro; 11.4 to 68.1%) and RS (starch undigested after 120 min in vitro; 3.1 to 85.0%; **Table 2.1**).

Table 2.1 Kinetics parameters of in vitro starch digestion and portal glucose appearance in pigs of four starch diets.

Characteristic	Kinetics of starch digestion				
	Rapidly	Moderately rapid	Moderately slow	Slowly	Pooled SEM
In vitro kinetics parameter[1]					
Plateau[2], % of starch	75.87	74.85	73.91	49.83	0.67
$K_{plateau}$[3], % of starch/min	1.92	1.02	0.38	0.22	0.02
$C_{plateau}$[4]	38.90	15.67	0.00	0.00	1.12
Peak time, min	14.49	9.16	0.03	0.05	1.34
Maximum rate of glucose release, %/min	1.06	0.73	0.38	0.22	0.01
In vivo kinetics parameter[5]					
Plateau[6], % of starch	48.1	51.6	46.89	31.4	5.42
$K_{plateau}$[7], % of starch/min	0.63	0.25	0.34	0.16	0.12
$C_{plateau}$[8]	86.5	22.2	34.30	8.7	3.81
Peak time, min	78.2	73.7	76.05	48.8	9.89
Maximum rate of glucose release, %/min	0.29	0.15	0.18	0.10	0.05
Starch composition[9]					
RDS, % of starch	28.8	19.7	7.1	3.6	0.12
SDS, % of starch	68.1	46.8	31.7	11.4	0.21
RS, % of starch	3.1	33.5	61.2	85.0	0.22

[1]Determined by in vitro enzymatic digestion until 480 min (4), values are means, $n = 3$; [2]Peak in vitro starch digestion; [3]Maximum rate of in vitro starch digestion; [4]Shape parameter of in vitro starch digestion; [5]Determined by glucose absorption until 480 min postprandial during present pig study, values are means, $n = 4$; [6]Peak net portal glucose appearance; [7]Maximum rate of net portal glucose appearance; [8]Shape parameter of net portal glucose appearance; [9]RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch; values are means, $n = 3$.

2.2.3 Animal experiment

The animal protocol was approved by the Animal Care and Use Committee for Livestock at the University of Alberta. Four female pigs (35.0 ± 0.2 kg initial BW) were catheterized in the portal vein and carotid artery and a flow probe was installed around the portal vein (Hooda et al., 2009). After recovery, pigs (43.2 ± 4.8 kg BW) were fed one of the four diets containing purified starches (**Table 2.2**) in a 4 x 4 Latin square design. Pigs were fed 1200 g/d in two equal meals at 0800

and 2000. The nutrient composition of the diets met NRC requirements (NRC, 1998). On d 7 of each period, blood samples from the portal vein and carotid artery catheters were collected 15 min before morning feeding and 0, 15, 30, 60, 120, 240, 360, and 480 min after feeding. During each blood collection, portal blood flow rate was measured using a flow meter (Transonic Systems Inc., Ithaca, NY). Plasma flow rate was calculated from blood flow using the equation; plasma flow = blood flow × (1 − hematocrit). Plasma was analyzed for glucose (Glucose oxidase kit; Diagnostics Chemicals Ltd., Charlottetown, Prince Edward Island, Canada).

Table 2.2 Ingredient compositions of diets.

Ingredient	g/kg of diet
Starch[1]	700
Casein[2]	140
Fish meal[3]	74
Cellulose[4]	40
Canola oil	10
Limestone	10
$CaHPO_4$	8
NaCl	3
Vitamin premix[5]	5
Mineral premix[6]	8
K_2CO_3	2

[1]Four sources, either: a) Remyline AX-DR rice (Remy Industries, Leuven-Wijgmaal, Belgium); and b) Remy B7 rice (Remy Industries); c) Nastar pea (Cosucra Groupe Warcoing, Warcoing, Belgium); d) Gelose 80 corn (Penford Food Ingredients, Centennial, CO) were used to prepare rapidly, moderately rapid, moderately slow, and slowly digestible starch diets, respectively; [2]Calcium caseinate, American Casein Company, Burlington, NJ; [3]Menhaden fish meal, Omega Protein, Hammond, LA; [4]Solka-floc, International Fiber Corp., North Tonawanda, NY; [5]Provided per kg diet: Zn, 100 mg as $ZnSO_4$; Fe, 80 mg as $FeSO_4$; Cu, 50 mg as $CuSO_4$; Mn, 25 mg as $MnSO_4$; I, 0.5 mg as $Ca(IO_3)_2$; Se, 0.1 mg as Na_2SeO_3; [6]Provided per kg diet: retinol, 2.5 mg; cholecalciferol, 20.6 µg; α-tocopherol, 2.7 µg; niacin, 35 mg; D-pantothenic acid, 15 mg; riboflavin, 5 mg; menadione, 4 mg; folic acid, 2 mg; thiamine,1 mg; D-biotin, 0.2 mg; vitamin B_{12}, 0.025 mg.

Glucose absorption was calculated using the formula q = (Portal vein concentration – Carotid artery concentration) × F (dt). Cumulative net glucose absorption was calculated subsequently using the formula Q = $\sum_{t0}^{t480} q$ (Rérat et al., 1984). In these formulae, q is the amount of portal glucose appearance within time period dt, F is plasma flow in the portal vein, and Q is the amount of glucose absorbed from time t_0 to t_{480} (0 to 480 min postprandial). Percentage of starch that is digested and absorbed as glucose per minute is calculated and fitted into a model using equations 1 and 2 (provided below).

2.2.4 Model used

Using the data obtained from in vitro and in vivo studies, the digestion coefficient of starch was calculated using the following formula: Digestion coefficient at time t = [glucose present at time t – 0 min glucose release) × 0.9]/total starch. A factor of 0.9 was used, because the MW of glucose as incorporated into starch is 90% of that of free glucose. The Chapman-Richards model describes a complex non linear function where an independent variable can undergo different rates of change such as initial slow rate followed by rapid progression up to a peak or maximum rate (Fekedulegn et al., 1999). In vitro starch digestion and portal glucose appearance in the present study are examples of such variables, and thus can be described by such a model. Hence, a modification of the Chapman-Richards model described by van Kempen et al. (van Kempen et al., 2007) (Eq. 1 and 2) was used to model in vitro starch digestion and glucose release and portal glucose appearance in the present study:

Starch hydrolysis % =

$$A + B \times (1 - \exp \times (\frac{-K_{plateau}}{Plateau} \times time))^{(\frac{Cplateau}{Plateau}+1)} \quad \text{(Eq. 1)}$$

Glucose release, %/min=

$$\frac{(\frac{C_{plateau}}{Plateau} + 1 \times ((1 - \exp \times (-K_{plateau} \times time))^{(Cplateau-1)} \times K_{plateau} \times \exp(-K_{plateau} \times time)}{0.9} \quad \text{(Eq. 2)}$$

Where, A is the free glucose present in the sample before enzyme addition, B is the glucose released by exhaustive digestion, $K_{plateau}$ is the rate of glucose release corrected for plateau effects, Plateau is the sum of A + B and thus the maximal glucose release as a % of sample weight, and $C_{plateau}$ is the sigmoidal/shape modifier corrected for plateau effects. As compared to standard Michaelis-Menten enzyme kinetics model, the modifications are that K is substituted by K corrected for plateau effects ($K_{plateau}$) and a shape parameter $C_{plateau}$ is added that is also corrected for plateau effects. These modifications were applied because digestion was observed to be sigmoidal for some starches and because the rate and shape parameters were affected by the amount of starch incubated, and the above correction eliminated this effect.

2.2.5 Correction of in vitro glucose release with gastric emptying

The link between the in vivo and in vitro data might improve with a correction of in vitro data for gastric emptying. Previously, gastric emptying has been explained by the formula: $y(t) = (1 - \exp^{-kt})^{\beta}$ (Camilleri et al., 1985; Siegel et al., 1988). In this equation, k is the gastric empty rate in min and β is the shape parameter. A β

> 1 indicates an initial delay in gastric emptying, and β < 1 indicates initial rapid emptying. Hence, the following equation was tested in this study:

In vitro starch hydrolysis corrected for gastric emptying =

$$D \times (1 - \exp^{-kt})^{\beta} \int (A + B \times (1 - \exp \times (\frac{-K_{plateau}}{Plateau} \times time))^{(\frac{Cplateau}{Plateau}+1)}) \quad \text{(Eq. 3)}$$

Where D is a multiplier for converting absolute in vitro to in vivo data. D can thus account for glucose consumption by the gastro-intestinal tract itself.

To test Eq. 3, 16 sequential time-periods were created so that for each time period an equal amount of starch left the stomach that each was assumed subsequently digested at the rates determined in vitro. By integrating these data across the 16 time-periods, a glucose appearance curve in portal blood was created and compared to the actual in vivo data. Using an iterative process while attempting to minimize the RMSE, values for D, k, and β were obtained.

2.2.6 Statistical analysis

The effect of starch diets on portal glucose appearance at different time points was analyzed using repeated measures in a mixed model of SAS (version 9.1; SAS Inst. Inc., Cary, NC). The model included diet, time, and their interaction as fixed effects and pig and period as random effects. The relationship of in vitro glucose release and portal glucose appearance was analyzed using the regression procedure of SAS. Least square means were used for all calculations and kinetics model development. Significance of difference was set at $P < 0.05$.

2.3 Results

2.3.1 In vitro starch digestion and glucose release

In vitro glucose release could be modeled effectively with equation 1 (R^2 = 0.998; **Figure 2.1**). Digestion curves also clearly exhibited a lag phase that could be described with the sigmoidal shape parameter. Specifically, across starches, 0.33% (range: 0.25 to 0.46%) of total starch was present as free glucose at 0 min after the start of incubation. In vitro glucose release peaked between 0 and 15 min after the start of incubation and the peak was highest and latest ($P < 0.01$) in the rapidly digestible starch diet, followed by moderately rapid, moderately slow, and slowly digestible starch diets (**Figure 2.1A**).

By 60 min, glucose release dropped drastically for the rapidly and moderately rapid digestible starch diet and from 180 min onward, the rate of glucose release was higher ($P < 0.01$) for moderately slow and slowly digestible starch than for rapidly and moderately rapid digestible starch diets. The modeled in vitro glucose release had reached 95% of the plateau value after 95 min for the rapidly digestible starch but the slowly digestible starch required 400 min of digestion to reach 95% of plateau. Cumulative starch digestion at 120 and 480 min was highest ($P < 0.05$) for rapidly (72.4 and 75.9%) followed by moderately rapid (63.0 and 74.9%), moderately slow (41.0 and 73.9%) and least for slowly (27.2 and 49.8%) digestible starch diets, respectively (**Figure 2.1B**). The ranking of cumulative starch digestion was consistent for the entire incubation among starch

sources. Out of total starch digested, 4.5 and 45.5% was digested from 120 to 480 min of the incubation, respectively, in rapidly and slowly digestible starch diets (**Figure 2.2**).

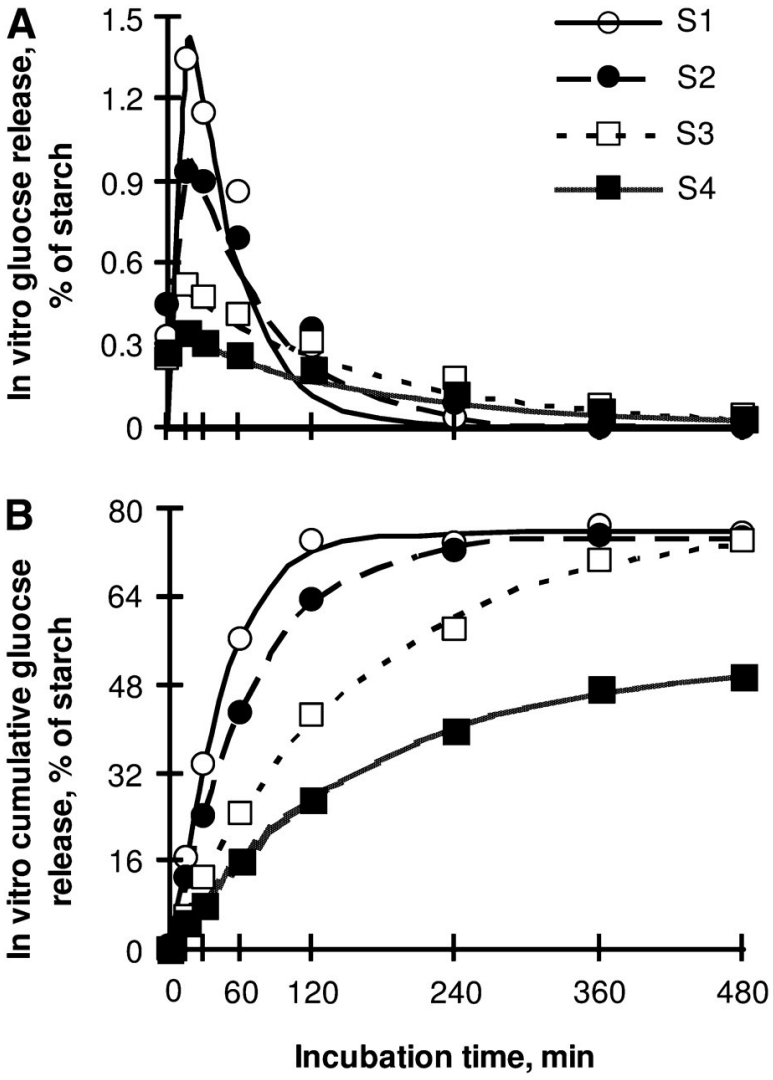

Figure 2.1 Absolute (**A**) and cumulative (**B**) release of glucose during in vitro digestion of 4 starch diets. A modified Chapman-Richards model was used to predict kinetics of glucose release from observed values (R^2 = 0.998). Values are means, n = 4. In A, the SE ranged from 0.00 to 0.02%; in B, the SE ranged from 0.02 to 2.03%.

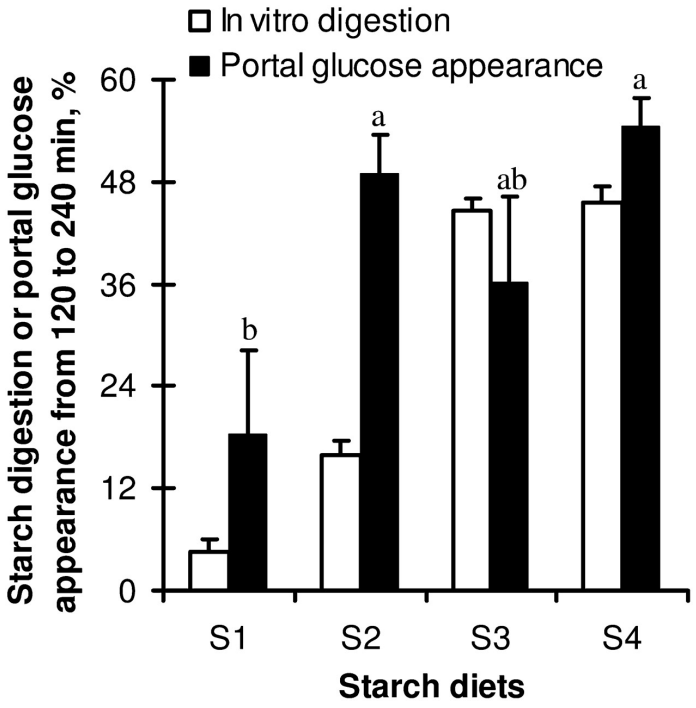

Figure 2.2 In vitro glucose release from 120 to 480 min of incubation and portal glucose appearance in pigs from 120 to 480 min after feeding from 4 starch diets. Values are means + SE, $n = 3$ (in vitro release) or 4 (portal appearance). For glucose appearance, means without a common letter differ, $P < 0.05$.

2.3.2 Portal glucose appearance

Net portal appearance of glucose peaked around 60 min after feeding the animals, and the peak was highest ($P < 0.05$) in rapidly digestible starch diets (0.24 %/min) followed sequentially by moderately rapid (0.17), moderately slow (0.16), and slowly (0.10) digestible starch diets (**Figure 2.3A**). Subsequently, glucose appearance dropped drastically for the rapidly digestible starch diet compared to other diets and from 270 min onward the rate of glucose release was numerically lower for the rapidly digestible starch diet compared to other diets. Cumulative portal appearance of glucose (% of starch) at 120 min after feeding was lower ($P < 0.01$) for slowly than rapidly digestible starch and at 480 min after feeding was

lowest ($P < 0.001$) for slowly compared to rapidly, moderately rapid, and moderately slow digestible starches (**Figure 2.3B**). In vivo glucose release could be modeled effectively with equation 1 ($R^2 = 0.964$). Out of the total glucose that appeared in portal circulation, 18.3 and 54.4% appeared from 120 to 480 min postprandial, respectively, in rapidly and slowly digestible starches (**Figure 2.2**).

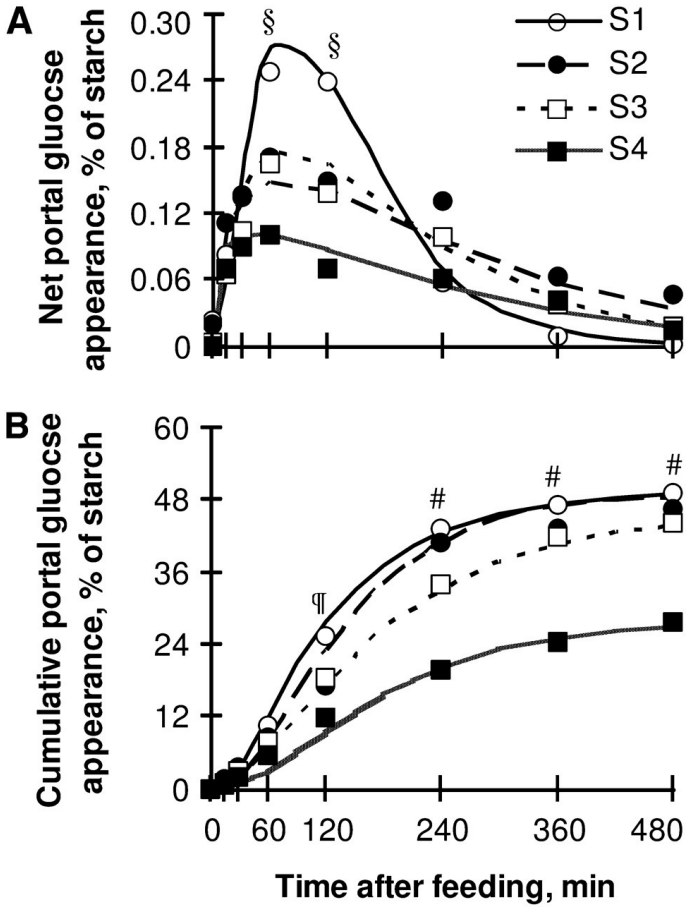

Figure 2.3 Absolute (**A**) and cumulative (**B**) portal appearance of glucose in pigs fed 4 starch diets. A modified Chapman-Richards model was used to predict kinetics of portal glucose appearance from observed values ($R^2 = 0.964$). Values are means, $n = 4$. In A, the SE ranged from 0.01 to 0.03; in B the SE ranged from 0.02 to 5.22%. Symbols indicate that means differ, $P < 0.05$: §, S1 > S2 = S3 > S4; ¶, S1 > S4; and #, S1 = S2 = S3 > S4.

2.3.3 Gastric emptying

The calculated time points at which equal amount of starch left the stomach were 0, 6.7, 13, 19.6, 26.5, 33.8, 41.8, 50, 60.1, 70.9, 83.4, 98, 115.8, 138.7, 170.8, 225.5, and 480 (fixed to end of measurements) min. D converged to 0.85, k to 0.0075, and β to 0.76, suggesting a half-time for gastric emptying of 69 min and a lag phase of 4.6 min, and rapid initial emptying.

2.3.4 Relation of in vitro starch digestion with portal glucose appearance

The in vitro starch digestion had a quadratic relationship with portal glucose appearance (R^2 = 0.89; 0.01x^2 + 0.07x + 0.53; P < 0.001; **Figure 2.4A**). The in vitro starch digestion corrected for gastric emptying had a linear relation with portal glucose appearance (R^2 = 0.95; y = 0.95x; P < 0.001; **Figure 2.4B**).

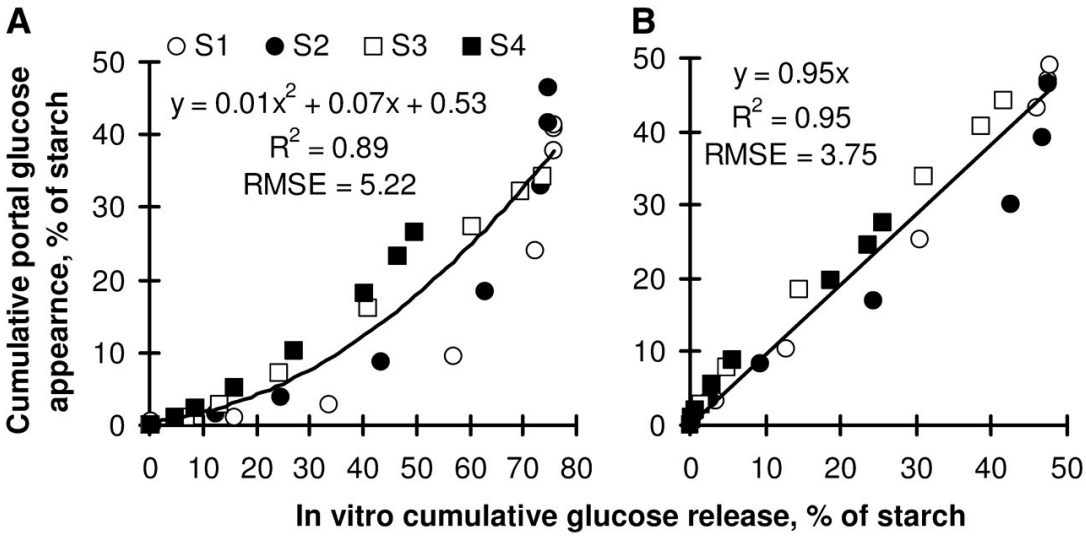

Figure 2.4 Prediction of modeled net portal glucose appearance in pigs fed 4 starch diets using in vitro cumulative glucose digestion (**A**) and in vitro cumulative glucose digestion corrected for predicted gastric emptying (**B**); RMSE = root mean square error.

2.4 Discussion

The current paradigm is that the in vitro-based starch fractions RDS, SDS, and RS reflect the in vivo glucose response (Englyst et al., 1992; Englyst et al., 1999). However, whether these fractions accurately translate to in vivo glucose absorption is controversial. For example, 30 min in vitro starch digestion of cooked rapidly and slowly digestible legume starch related weakly (R^2 = 0.41) with 0 to 60 min postprandial glucose responses (O'Dea and Wong, 1983). Glycemic index was moderately related with rapidly (R^2 = 0.54) and slowly digestible starch (R^2 = 0.63) from cereal products (Englyst et al., 2003). Similarly, in vitro digestion was related (R^2 = 0.76) with rate of digestion in chickens fed cereal starches (Weurding et al., 2001). Finally, RS of rice, cornflakes and baked beans measured after 15 h but not after 120 min in vitro digestion was related strongly (R^2 = 0.94) to in vivo RS in ileostomy patients (Muir and O'Dea, 1993). In vitro predictions of in vivo glucose release thus vary with method and starch source (Champ, 1992; Englyst et al., 1992; Muir and O'Dea, 1993). Hence, an important proportion of the variation between in vitro starch digestion and in vivo glucose response is yet to be defined and should be considered to improve the accuracy of prediction equations.

Possible explanations for these discrepancies in previous studies between in vitro starch digestion and in vivo glucose response include: a) confounding effects of nutrients other than starch present in grains and diets; b) coverage of limited postprandial duration (typically 120 min); c) use of discrete glucose response

observations as opposed to continuous glucose kinetics response curves; and d) observation of glucose response in peripheral circulation as opposed to portal circulation.

The present study was designed to avoid these shortcomings. First, possible confounding effects of starch-associated compounds such as protein, fat, and fiber were avoided by replacing unpurified starch in diets (Thorne et al., 1983; Annison and Topping, 1994) with purified starch. Second, in vivo and in vitro responses were extended beyond 120 min because a significant proportion of in vitro glucose release and portal glucose appearance takes place after 120 min in our and other studies (Muir and O'Dea, 1993). Third, to measure in vivo glucose response, postprandial net portal glucose appearance replaced peripheral glucose (O'Dea and Wong, 1983; Priebe et al., 2008) because the latter does not account for hepatic glucose consumption (Woerle et al., 2003), and, thus, under-represents true glucose uptake. Fourth, discrete glucose response observations were converted to continuous glucose kinetics response curves using a modified Chapman-Richards model (van Kempen et al., 2007).

Specifically for this last point, glucose data were modeled to facilitate the comparison between in vivo and in vitro. For this type of work, typically the first-order derivative of an association-type exponential model to determine the kinetics of starch digestion is used: Starch hydrolysis = plateau × (1 – exp(–K × time)). This model is based on the rate and maximum release of glucose.

However, this model poorly fit the digestion curves observed in our study. First, this model does not represent the sigmoidal digestion pattern that was observed especially for rapidly digesting starch sources like wheat (van Kempen et al., 2007). A possible explanation for the sigmoidal digestion is that starch granules with type A crystalline patterns are digested inside-out (MacGregor and Ballance, 1980). In vivo, the sigmoidal pattern is also reinforced by an initial delay in digesta transit (Siegel et al., 1988; Ziessman et al., 1992). Second, the model does not consider the amount of free glucose initially present in the sample. The present study finding that samples contained up to 0.46% of glucose at the start of incubation with pancreatin justified the inclusion of initial glucose data in the model. The modified Chapman-Richards model uses the parameters of the first-order model and also describes the sigmoidal nature of starch digestion and initial glucose release. Thus, this model was used to determine the kinetics of in vitro starch digestion and net portal glucose appearance.

Portal glucose appearance was strongly related with in vitro starch digestion (R^2 = 0.89; **Figure 4A**), a substantial improvement over most of the studies listed above. However, the relation was not linear, indicating that an important variable was missing and also had a high prediction error (RMSE = 5.22). The resultant relationship was thus not satisfactory.

A major discrepancy between in vivo and in vitro digestion is that in vitro the entire sample is incubated with an optimized enzyme cocktail at time 0. In

contrast, in vivo a meal is eaten over time (minutes) and subsequently the stomach acts as a buffer that releases the content slowly and non-linearly (hours). Released digesta are mixed with pancreatic amylase, which is slowly and semi-continuously released under complex physiological control, and brush-border disaccharidases (Liddle, 2006). Finally, glucose present in the incubation solution determines in vitro glucose release, whereas glucose released in the intestinal lumen has to pass across the gut epithelium before reaching the portal vein (minutes). Glucose transport across the epithelium will delay appearance while epithelial glucose use will reduce portal appearance of glucose (Huntington et al., 2006). Indeed, the initial lag phase was longer in vivo and maximal glucose release rates occurred roughly 1 hour later than in vitro. The subsequent decline was also notably slower in vivo indicating a longer half-life. A logical extension for the above model is thus to take the digestive tract prior to the portal vein into consideration. Gastric emptying is likely the dominant factor as it occurs over hours rather than minutes and hence was the focus for a correction factor.

Indeed, correction of in vitro data with gastric emptying yielded a much improved and linear fit between in vivo and in vitro data. The obtained gastric emptying parameters in the present study are in perfect agreement with values observed by Lefebvre et al. (2005) who obtained a halftime of 74 min and a lag phase of 3 min in pigs of comparable size. We assumed that the four starch sources had an equal rate of gastric emptying, but realize that variation may exist; determination of actual gastric emptying will likely further improve the prediction accuracy.

The D value of 0.85 indicates that 15% less glucose is recovered in vivo than in vitro, in line with differences between in vivo and in vitro plateau values. The likely explanation for this difference is glucose consumption by the intestinal microbiota and epithelium. Glucose is certainly not the preferred energy substrate of the epithelium, but is nevertheless used as a fuel (Windmueller and Spaeth, 1980; Wu et al., 1995). The correction of in vitro starch digestion for gastric emptying and intestinal glucose utilization thus improved the relation between in vitro starch digestion and portal glucose appearance, decreased the prediction error, and changed the relationship from quadratic to linear. This provides strong mathematical evidence that gastric emptying and intestinal glucose utilization should be considered when extrapolating in vitro to in vivo data.

Resistant starch is defined as the sum of starch and starch-degradation products that reach the large intestine of monogastric species and is predicted by Englyst et al. (Englyst et al., 1992) as the amount of starch not digested after 120 min of incubation. A cut-off of 120 min is supported by data from Englyst et al. (1996a), whom showed that 100% of in vitro determined RS from retrograded cereals was recovered in ileal digesta in humans. Conflicting with this observation, in the present study, a significant proportion of starch digestion occurred from 120 to 480 min (in vitro, up to 45.5% of total glucose release; in vivo, up to 54.4% of total glucose appearance in portal circulation). Similar to the present study, in vitro RS from up to 360 min digestion was related poorly with in vivo RS in humans eating diets containing 0.7 to 5.7% RS from ground rice, cornflakes and

baked beans (Muir and O'Dea, 1993). These findings indicate that the 120 min digestion values are inappropriate as a general recommendation for determining resistant starch. Instead, by extrapolating in vitro digestibility data to infinity (plateau values), a good relation is obtained between in vivo and in vitro (R^2 = 0.95 in our study). Practically, 95% of starch was digested in vitro by 400 min for even the slowly digestible starch. Hence, in vitro incubations of 400 min or more, or e.g., 16 h as described by McCleary et al. (2002) are all adequate for determining RS if modeling of the data is not feasible. In vivo plateau values, though, were 13% lower across samples, indicative of intestinal consumption of glucose.

The RDS is commonly seen as the fraction with the strongest impact on blood glucose and insulin responses after eating a starch source, and is linked with type-II diabetes. If starch digestion was non-sigmoidal, this interpretation would be warranted. However, findings from the present study and van Kempen et al. (2007) indicate that starch digestion is sigmoidal in nature, especially sources high in RDS. Maximum rates of starch digestion were observed as late as 20 min into the in vitro assay (T. A. T. G. van Kempen, unpublished data); therefore, defining the first 20 min of in vitro digestion as RDS is inappropriate. The definition of SDS, digested between 20 and 120 min or between RDS and RS, also loses significance if both RDS and RS are not defined properly. The substantial proportion of starch digestion and glucose absorption that occurs between 120 to 480 min postprandial indicates that the glycemic index, which is

derived by measuring blood glucose from 0 to 120 min postprandial, has little biological relevance and cannot properly differentiate foods with different rates of starch digestion. Our proposal is to instead model digestibility curves using the modified Chapman-Richards model. This allows for the calculation of the maximal rate of starch digestion and thus glucose release as a predictor of insulin response, the lag time for this peak, the final extent of digestion, and the kinetics of starch digestion throughout.

In conclusion, starch digestion can be modeled accurately using the modified Chapman-Richards model that correctly describes the sigmoidal digestion curves observed in vivo and in vitro. In vivo portal glucose appearance data can be predicted effectively from in vitro digestibility data after correction for gastric emptying and correction for intestinal glucose utilization. The resultant model explained 95% of the in vivo variation when testing 4 heterogeneous starch sources. The fractions RDS, SDS, and RS appear to have less biological relevance and are thus of little value for predicting the in vivo responses to starch sources.

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Chapter 3. Starch with high amylose and low in vitro digestibility increases short-chain fatty acid absorption, reduces peak insulin secretion, and modulates incretin secretion in pigs[1]

3.1 Introduction

Starch is the major dietary source of glucose for monogastric species. The kinetics (rate and extent) of starch digestion depends on starch chemistry, particle size, processing method, and association with other compounds such as lipids, protein, fiber, minerals, and anti-nutritional factors (Singh et al., 2010). Among starch chemistry characteristics, amylose content and crystallinity are important factors affecting starch digestibility and thus metabolic responses in vivo. Unlike amylopectin that is highly branched, amylose polymers have less surface area and more intra-molecular hydrogen bonds (Singh et al., 2010) and can form complexes with surface compounds such as fatty acids (Cui and Oates, 1999). Thus, amylose polymers are digested at a lower rate and extent than amylopectin polymers due to decreased accessibility for α-amylase to the molecule. Conversely, the crystalline region of starch granules, which is formed by densely packed glucose molecules mainly from amylopectin, has lower accessibility for enzymes compared to the amorphous region and thus, purportedly lower digestibility (Singh et al., 2010).

[1]A version of this chapter has been published: Regmi, P. R., T. A. T. G. van Kempen, J. J. Matte and R. T. Zijlstra. 2011. Starch with high amylose and low in vitro digestibility increases short-chain fatty acid absorption, reduces peak insulin secretion, and modulates incretin secretion in pigs. J. Nutr. 141:398-405.

The kinetics of starch digestion influence postprandial glucose and insulin responses that are associated with the risk of metabolic diseases such as type II diabetes in human (Mathers and Daly, 1998). In addition, luminal glucose and SCFA, affected by starch digestibility, stimulate the secretion of incretins such as glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) (Baggio and Drucker, 2007). Increased blood glucose concentration stimulates insulin secretion, and incretins potentiate glucose-stimulated insulin secretion (Baggio and Drucker, 2007). Although effects of amylose (Behall and Hallfrisch, 2002; Deng et al., 2009) and resistant starch (RS) (Raben et al., 1994; van der Meulen et al., 1997; Deng et al., 2009) on peripheral glucose and insulin response have been reported, the association that allows starch chemistry to affect the absorption of starch-derived nutrients and secretion of insulin and incretins is not clear. In addition, the relationship between the kinetics of nutrient absorption and hormone secretion is not clear.

Therefore, diets containing 4 purified starches with a wide difference in starch chemistry were consumed by porto-arterial catheterized pigs to test the hypothesis that a higher content of amylose reduces glucose absorption, insulin and incretin secretion and increases SCFA absorption via a lower starch digestibility. The objectives were to a) determine the role of starch chemistry on the kinetics of starch-derived nutrient absorption and insulin and incretins secretion and b) to understand the interrelationship of the kinetics of nutrients absorption and insulin and incretin secretion as affected by starch chemistry.

3.2 Materials and Methods

3.2.1 Starch characterization

Remyline AX-DR 0% amylose rice starch (Remy Industries, Leuven-Wijgmaal, Belgium), Remy B7 20% amylose rice starch (Remy Industries), Nastar 35.5% amylose pea starch (Cosucra Group Warcoing, Warcoing, Belgium), and Gelose 80% amylose corn starch (Penford Food Ingredients, Centennial, CO) samples were used in the study (**Table 3.1**). Starch content was determined after enzymatic hydrolysis of samples using thermostable α-amylase and amyloglucosidase (Megazyme Int. Ireland Ltd., Wicklow, Ireland). Amylose content was determined using a Megazyme amylose / amylopectin assay kit (Megazyme Int.). Starch granule size was determined using a scanning-electron microscope (6301F, J*EOL*, Tokyo, Japan) (**Figure 3.3**). Crystallinity was determined after X-ray diffraction of starches (Geigerflex 2173, Rigaku/MSC, The Woodlands, TX) using a cobalt X-ray source (**Figure 3.1**) (Nara and Komiya, 1983). Crude protein content was determined using an N-analyzer (FP-2000 N Analyzer; Leco Instrument Inc., St. Joseph, MI). Crude fat content was determined by extraction with ether (method 920.39; AOAC, 1990) using a Goldfisch Fat Extractor (Laconco Corp., Kansas City, MO).

Table 3.1 Characteristics of 4 starches used in the diets

	Starches			
Characteristic (DM basis)	S1	S2	S3	S4
Starch, *g/kg*	963	958	979	943
Maximum rate of in vitro digestion,[1] *g/kg · min*	10.6	7.3	3.8	2.2
Total in vitro starch digestion,[1] *g/kg*	759	749	739	498
Free glucose,[2] *g/kg*	2.3	2.5	1.6	1.8
Amylose content, *g/kg*	< 50[4]	196	284	632
Crystallinity,[3] *%*	40	36	30	24
Starch granule size				
Width, μm	1.8 – 3.8	2.4 – 5.6	5.0 – 13.5	3.4 – 10.9
Length, μm	2.4 – 5.7	2.9 – 8.9	5.4 – 34.0	3.5 – 16.6
Crude protein, *g/kg*	17	18	18	17
Crude fat, *g/kg*	5.1	5.6	3.3	4.1
Ash, *g/kg*	1.8	2.8	0.4	1.0

[1]Determined by 8 h in vitro enzymatic digestion (van Kempen et al., 2010); [2]0 min glucose release (van Kempen et al., 2010); [3]Determined by X-ray diffraction (**Figure 3.1**), and can be visualized as opaque area of starch particle observed under transmission electron microscopy (**Figure 3.2**); [4]Detection limit was 50 g/kg.

The starches were considered rapidly digestible (S1), moderately rapid digestible (S2), moderately slow digestible (S3), and slowly digestible (S4) based on the maximum rate of in vitro starch digestion based on a two-stage in vitro digestion assay modified after Englyst (S1, 1.06; S2, 0.73; S3, 0.38 and S4, 0.22%/min van Kempen et al., 2010).

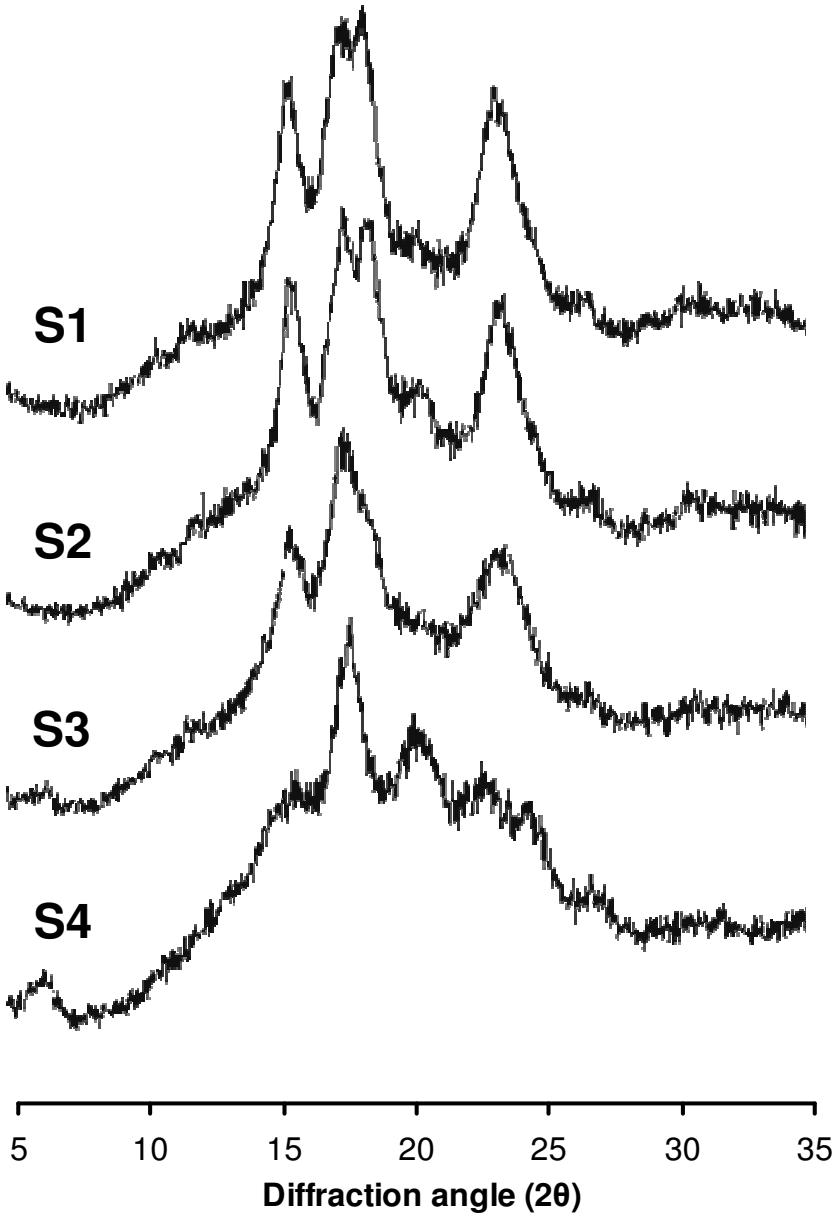

Figure 3.1 X-ray diffraction pattern of 4 starches

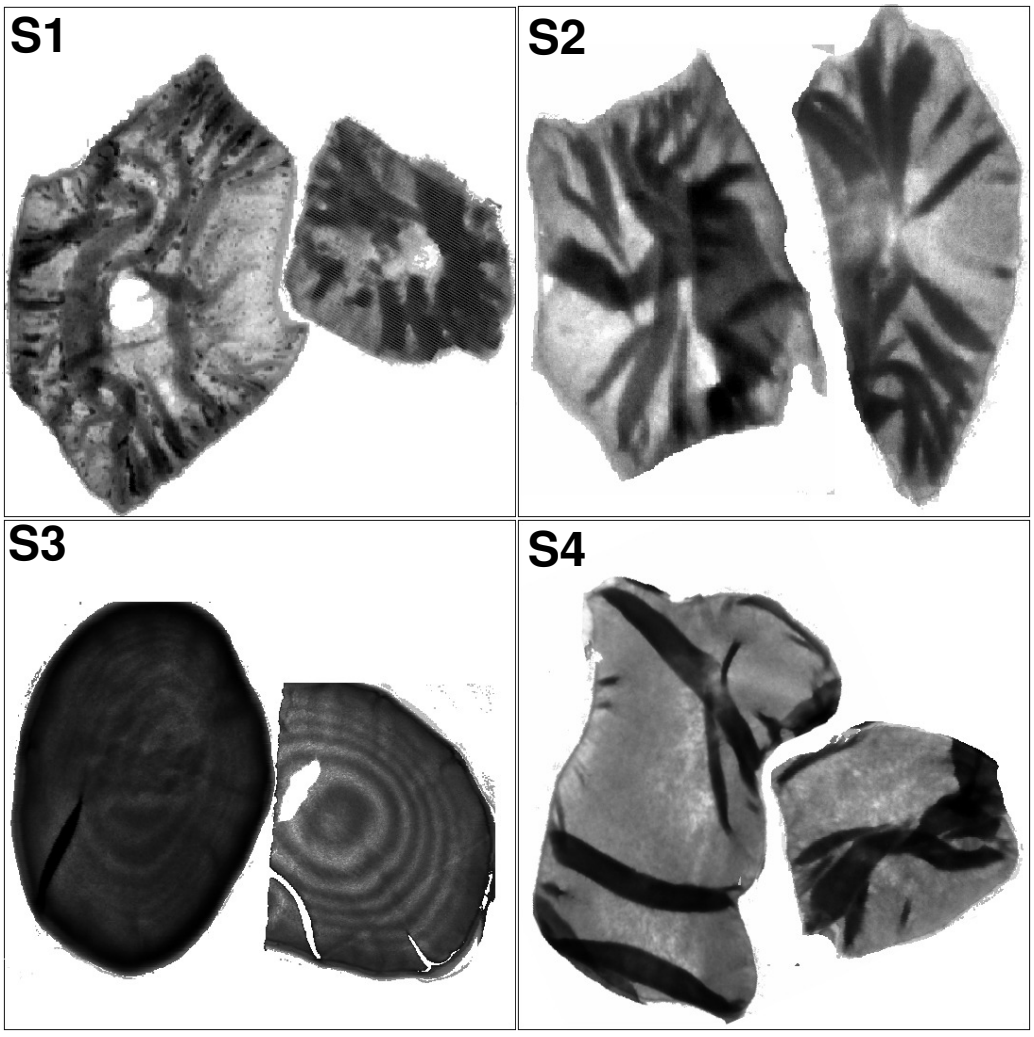

Figure 3.2 Transmission electron micrograph of 4 starches.

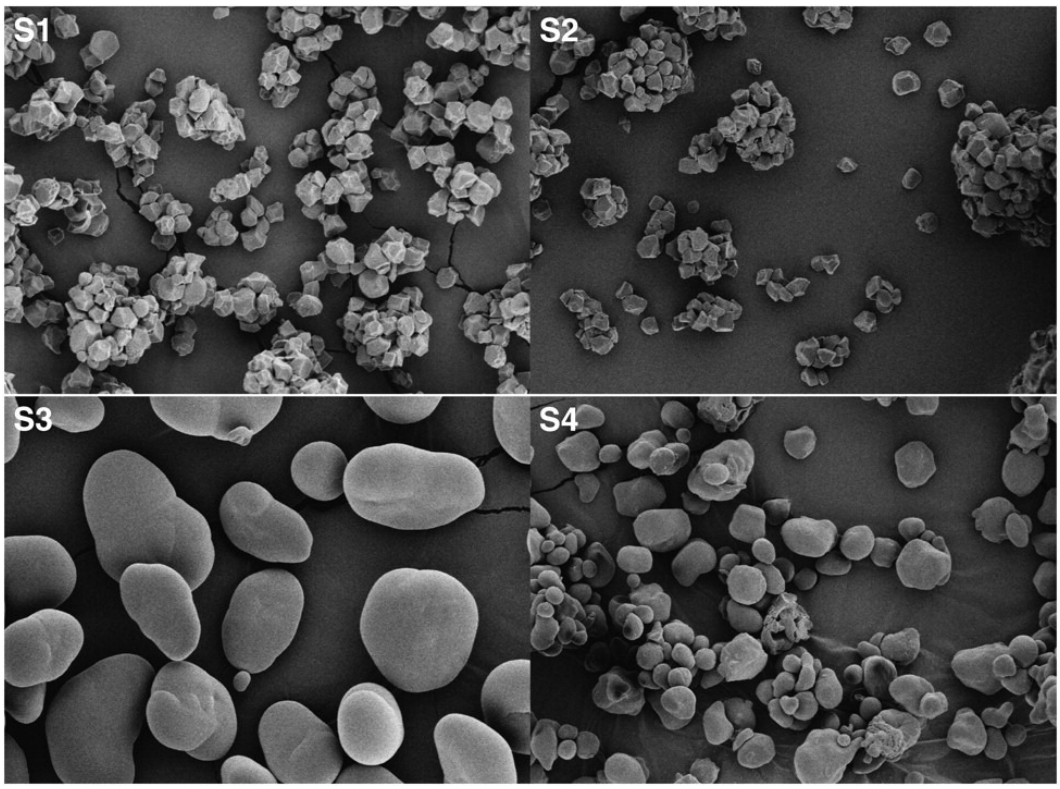

Figure 3.3 Electron micrograph of the 4 starches (×350).

3.2.2 Animal experiment

The animal use protocol was approved by the Animal Care and Use Committee for Livestock at the University of Alberta. Four female pigs were surgically modified (Hooda et al., 2009) and consumed one of the four diets containing 70% of the purified starches (**Table 2.2**) in a 4 × 4 Latin square design. Blood sampling, portal blood-flow rate measurements and plasma flow rate calculations were described previously (Chapter 2) and the same samples were used for the present study.

3.2.3 Plasma analysis and calculations

Plasma was analyzed for glucose (intra- and inter-assay CV ≤ 2.0; Glucose oxidase kit; Diagnostics Chemicals Ltd., Charlottetown, Prince Edward Island, Canada), L-lactate (intra- and inter-assay CV ≤ 2.0; lactate dehydrogenase-based kit; State University of New York, Buffalo, NY), and SCFA (acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, and caproate) using GC (intra- and inter-assay CV ≤ 11.2) as described by Brighenti (Brighenti, 1997) with some modifications. Briefly, isocaproic acid (0.93 mg per L of water) was used as an internal standard (12.5 µL/100 µL of plasma) and plasma was deproteinized using 25% phosphoric acid solution (8 µL/100 µL of plasma) at 60ºC for 30 min. The solution was centrifuged at 8000 g for 30 min to remove the proteins. The supernatant was analyzed for SCFA by GC using He as a carrier gas, a 30 m × 0.53 mm × 1 µm size column, and an FID detector (Hewlett Packard, Waldbroom, Germany). Insulin (intra-assay CV = 7.3 and inter-assay CV = 8.8) and C-peptide (intra-assay CV = 6.6 and inter-assay CV = 9.5) were analyzed by using porcine RIA kits (Linco, St. Louis, MO). For GIP analysis, 85 µg of aprotinin (Roche Diagnostics, Laval, Quebec, Canada) was added to each mL of plasma before storage to prevent GIP degradation. GIP content in the plasma was determined (intra-assay CV = 10.0 and inter-assay CV = 13.5) by RIA (Peninsula Laboratories, Belmont, CA). For GLP-1 determination, the samples were extracted using alcohol and determined (intra-assay CV = 12.8 and inter-assay CV = 15.1) by RIA (Peninsula Laboratories). As the half life of active GLP-1 in

plasma is very short (2 to 5 min), total GLP-1 were determined which may contain both active and inactive GLP-1.

Net portal appearance (NPA) of glucose, L-lactate, SCFA, insulin, C-peptide, GIP, and GLP-1 was calculated using the formula: NPA of nutrients or hormones = (portal concentration – arterial concentration) × plasma flow rate in the portal vein. Subsequently, cumulative 12-h NPA of nutrients was calculated. Peak NPA (C_{max}) and time to reach peak NPA (T_{max}) for glucose, insulin and C-peptide and time required to halve the peak NPA ($½T_{Cmax}$) for insulin and C-peptide were calculated. Carotid plasma glucose half-life (time required to halve the peak concentration of glucose in carotid plasma), which reflects insulin effects on blood glucose concentration, was also calculated. The simultaneous glucose uptake was not separated from the arterial glucose pool. Area under the curve (AUC) was calculated based on the trapezium rule using the formula: AUC = $\frac{1}{2}\sum_{i=0}^{n-1} (t_{i+1} - t_i)(y_i+y_{i+1})$. In the formula, t_i is a specific time point and y is the measurement (Matthews et al., 1990).

The total NPA of starch-derived nutrients such as glucose, L-lactate and total SCFA as a % of starch consumed was calculated. NPA of glucose was multiplied by a factor of 0.9 because the molecular weight of glucose as incorporated into starch is 90% of that of free glucose. Two moles each of L-lactate are considered to be produced from each mole of glucose (Kreisberg et al., 1971). As NPA of SCFA is primarily from microbial fermentation of undigested starch and protein

in the ileum and large intestine, NPA of SCFA from protein was deducted from the total NPA of SCFA to calculate NPA of SCFA from starch. NPA of SCFA from protein was calculated using NPA of branched chain fatty acids (BCFA), isovalerate and isobutyrate, as a marker because BCFA are solely produced from protein sources and 20% of the total SCFA production from protein was assumed to be BCFA (Macfarlane et al., 1992). Each mole of glucose is considered to be converted to either 2 moles each of acetate, propionate, and isopropionate or either 1 mole each of butyrate, valerate, and caproate (Louis et al., 2007).

3.2.4 Modeling of kinetics of insulin secretion

A Chapman Richards model was used to describe the kinetics of NPA of insulin based on measured insulin values. A modified model, corrected for plateau effect, was used to describe the NPA of glucose kinetics based on measured glucose values (van Kempen et al., 2010). Several models were evaluated for predicting NPA of insulin based on NPA appearance of glucose, but none yielded satisfactory results (data not shown). Instead, NPA of insulin was modeled based on in vitro glucose release using a linear model: Predicted NPA of insulin, pmol/mi*n* = A × in vitro glucose release corrected for gastric emptying, % of starch, where, A is a constant used to make the in vitro glucose release as the same scale as NPA of glucose in mmol/min.

3.2.5 Statistical analysis

NPA of nutrients, insulin, and incretins were analyzed using repeated measures in SAS (version 9.1; SAS Inst. Inc., Cary, NC). The model included diet, time, and

interaction of diet and time as fixed effects and pig and period as random effects. Pre-prandial observations were used as covariates to determine postprandial effects. C_{max}, T_{max} and $½T_{Cmax}$ for glucose, insulin and C-peptide were analyzed using the mixed model in SAS with diet as a fixed effect and period as a random effect. Means were separated for diets using the PDIFF statement in the Mixed model for individual time points after detecting a significant diet effect using SLICE/time. Pigs were considered the experimental units and significance of difference was set at $P < 0.05$. All variables were tested by the Kolmogoroff-Smirnoff test for normal distribution. Values in the text are means ± SEM.

Principal component (PC) analysis was performed using JMP (version 8.0.2; SAS Inst.). The loading plot of PC 1 and PC 2, the first 2 eigenvalues, were used to determine the correlation among NPA of nutrients and hormones and starch characteristics. The angles between the lines were used to describe the interrelationship. Simple linear and multiple linear regression analyses were performed using JMP to determine relationships between and among variables. Log transformed data were used for regression analyses to meet the assumption of linear relationships.

3.3 Results

All pigs were healthy throughout the experiment as observed by normal appetite and growth. Development of collateral circulation in the portal system was not observed during autopsy at the end of experiment. The blood flow rate was 1.2 ±

0.09 L/min and did not differ ($P > 0.05$) when pigs consumed the different starch diets. Blood flow rate was higher ($P < 0.05$) during 0 to 4 h than 4 to 10 h, followed by 10 to 12 h postprandial (data not shown).

3.3.1 Glucose

When pigs consumed the S4 diet, the peak (**Table 3.2**) and 12 h NPA (**Table 3.3; Table 3.4**) of glucose was lower ($P < 0.05$) than when they consumed the other 3 diets. The time point for peak NPA of modeled glucose did not differ ($P > 0.05$) among pigs consuming the 4 starch diets and ranged from 63 to 92 min postprandial. The time required to halve the peak concentration of glucose in the carotid artery was shorter ($P < 0.05$; **Table 3.2**) when pigs consumed the S1 diet than when they consumed the S4 diet.

Table 3.2 Kinetics parameters of NPA of glucose, insulin, and C-peptide in pigs consuming 4 starch diets differing in rate of digestion[1]

Kinetics parameter	Starch diets				Pooled
	S1	S2	S3	S4	SEM
Glucose					
C_{max},[2] *mmol*	6.61[a]	4.90[a]	5.12[a]	2.75[b]	0.63
T_{max},[3] *min*	62.6	92.3	77.6	67.5	21.3
Carotid plasma half-life,[4] *min*	380[b]	375[ab]	565[ab]	615[a]	99.4
Insulin					
C_{max}, *μmol*	0.91[a]	0.57[ab]	0.44[ab]	0.34[b]	0.220
T_{max}, *min*	47.4	75.5	97.4	143	40.7
½T_{Cmax},[5] *min*	62.7	82.6	112	113	47.3
C-peptide					
C_{max}, *μmol*	1.68[a]	1.48[ab]	1.04[ab]	0.87[b]	0.33
T_{max}, *min*	50.6	71.2	80.5	109.7	39.8
½T_{Cmax}, *min*	83.8	95.4	124	175	43.5

[1]Values are means and pooled SEM; $n = 4$. Means in a row without a common superscript differ, $P < 0.05$.

Table 3.3 NPA of major starch-derived nutrients in 12 h in pigs consuming 4 starch diets differing in rate of digestion[1]

	Starch diets				Pooled
Starch-derived nutrients	S1	S2	S3	S4	SEM
Total,[2] *% of starch consumed*	64.8	69.9	69.1	66.7	11.0
Glucose	49.7[a]	52.2[a]	49.6[a]	33.8[b]	8.6
L-lactate	9.17	11.1	10.6	12.3	2.44
Total SCFA	5.97[b]	6.59[b]	8.87[b]	20.5[a]	1.25
Acetate	5.46[c]	4.78[c]	7.66[b]	11.2[a]	0.738
Propionate	0.123[b]	1.37[b]	0.656[b]	5.65[a]	0.285
Butyrate	0.222[b]	0.186[b]	0.320[b]	2.51[a]	0.264
Molar proportions, *mol/100 mol*					
Acetate	89.4[a]	84.4[a]	86.8[a]	57.8[b]	3.13
Propionate	8.24[b]	12.2[b]	10.7[b]	29.5[a]	5.15
Butyrate	2.36	3.38	2.50	12.6	4.79

[1]Values are means and pooled SEM; $n = 4$; means in a row without a common superscript differ, $P < 0.05$. [2]The NPA of starch-derived nutrients was calculated as a percentage of starch consumed using the following assumptions: glucose in starch = NPA of (free) glucose × 0.9; 1 mol of glucose = 2 mol of L-lactate; NPA of SCFA from starch = total NPA of SCFA – NPA of SCFA from protein; NPA of SCFA from protein = (isovalerate + isobutyrate) × 5; 1 mol of glucose = either 2 mol each of acetate, propionate, and isopropionate or either 1 mol each of butyrate, valarate, and caproate.

Table 3.4 Portal and arterial plasma concentrations (averaged over 0 to 720 min after feeding) and 12 h NPA of nutrients, insulin, C-peptide, and incretins in pigs consuming 4 starch diets differing in rate of digestion[1]

	Carotid artery					Portal vein				
Item	S1	S2	S3	S4	SEM	S1	S2	S3	S4	SEM
Blood flow, *L/min*	-	-	-	-	-	1.24	1.17	1.17	1.22	0.053
Glucose, *mmol/L*	5.96	6.15	5.87	5.46	0.234	8.21^{a}	8.52^{a}	7.86^{a}	6.66^{b}	0.352
NPA of glucose, *mol*	-	-	-	-	-	0.90^{a}	1.09^{a}	0.89^{a}	0.54^{b}	0.157
L-lactate, *mmol/L*	2.30	2.33	1.95	2.15	0.481	3.04	3.47	2.60	2.91	0.606
NPA of L-lactate, *mol*	-	-	-	-	-	0.39	0.47	0.46	0.51	0.103
Insulin, *pmol/L*	186	158	136	105	29.2	365	300	273	207	52.4
NPA of insulin, *µmol*	-	-	-	-	-	85.0^{a}	78.5^{ab}	65.3^{ab}	50.0^{b}	17.4
C-peptide, *pmol/L*	239^{a}	263^{a}	207^{b}	160^{b}	38.6	424	469	361	327	73.6
NPA of C-peptide, *µmol*	-	-	-	-	-	162^{a}	155^{ab}	130^{ab}	107^{b}	23.5
GIP, *pmol/L*	128	119	115	123	8.91	173	126	152	149	59.6
NPA of GIP, *µmol*						25.5	16.6	21.8	18.4	6.41
GLP-1, *pmol/L*	2.43	2.50	2.76	2.64	0.352	3.62	3.46	5.07	4.34	0.631
NPA of GLP-1, *µmol*	-	-	-	-	-	0.752^{b}	0.746^{b}	1.61^{a}	1.40^{a}	0.223
Total SCFA, *mmol/L*	0.23	0.29	0.18	0.27	0.034	0.75^{c}	0.70^{c}	0.97^{b}	1.77^{a}	0.030
NPA of total SCFA, *mmol*	-	-	-	-	-	281^{c}	282^{c}	423^{b}	950^{a}	37.9
Acetate, *mmol/L*	0.20	0.27	0.17	0.23	0.030	0.66^{c}	0.60^{c}	0.82^{b}	1.04^{a}	0.017
NPA of Acetate, *mmol*	-	-	-	-	-	251^{c}	215^{c}	355^{b}	506^{a}	75.0
Propionate, *µmol/L*	14.4	13.5	8.78	11.2	7.04	33.1^{b}	101^{b}	86.3^{b}	401^{a}	64.2
NPA of propionate, *mmol*	-	-	-	-	-	6.21^{b}	61.4^{b}	30.4^{b}	255^{a}	27.2
Butyrate, *µmol/L*	2.00	0.61	3.42	19.7	4.50	21.7^{b}	60.4^{b}	25.7^{b}	196^{a}	41.4
NPA of butyrate, *mmol*	-	-	-	-	-	10.2^{b}	8.37^{b}	14.9^{b}	113^{a}	12.5
Valerate, *µmol/L*	0.214	0.461	0.308	0.613	0.104	4.41^{b}	5.08^{b}	8.19^{b}	38.5^{a}	2.07
NPA of valerate, *mmol*	-	-	-	-	-	1.94^{b}	4.70^{b}	3.81^{b}	25.8^{a}	0.650
Caproate, *µmol/L*	1.61	0.57	1.64	2.61	0.678	12.0^{b}	11.5^{b}	14.6^{b}	42.4^{a}	9.30
NPA of caproate, *mmol*	-	-	-	-	-	5.63^{b}	6.61^{b}	7.01^{b}	25.6^{a}	2.52
Branched chain fatty acid, *µmol/L*										
Isobutryate, *µmol/L*	2.52	1.89	2.35	3.03	0.656	9.02^{b}	6.34^{b}	11.0^{b}	17.9^{a}	1.39
NPA of isobutyrate, *mmol*	-	-	-	-	-	3.53^{b}	3.16^{b}	4.72^{b}	8.81^{a}	0.338
Isovalerate, *µmol/L*	0.879	1.03	0.840	0.856	0.913	11.6^{c}	8.41^{c}	16.4^{b}	30.2^{a}	1.46
NPA of isovalerate, *mmol*	-	-	-	-	-	4.67^{c}	4.08^{c}	7.15^{b}	15.6^{a}	0.459

[1]Values are means and SEM, $n = 4$, means in a row with superscripts without a common letter differ, $P < 0.05$.

When pigs consumed the S4 diet, NPA of glucose was lower ($P < 0.05$) than when they consumed the S1 diet from 45 min to 3.5 h, than when they consumed the S2 diet at 30 and 45 min and at 1.5, 2, and 3.5 h, and than when they consumed the S3 diet at 2 and 3.5 h postprandial (**Figure 3.4A**). Similarly, NPA of glucose was lower ($P < 0.05$) when pigs consumed the S3 diet than when they consumed the S1 diet at 45 min and 1.5 and 2 h postprandial, and was lower ($P < 0.05$) when pigs consumed the S2 diet than when they consumed the S1 diet at 1 and 2 h postprandial. NPA of glucose was negative from 8 to 12 h postprandial when pigs consumed the S1 diet and at 12 h when they consumed S2 and S3 diets and was positive for all time points when pigs consumed the S4 diet (**Figure 3.4A**). The NPA of glucose differed among four postprandial periods: 0 to 2 h (increased and peaked), 2 to 4 h (declined drastically), 4 to 10 h (declined further towards zero) and 10 to 12 h (negligible); hence, these time periods were used to calculate the AUC of NPA of nutrients and hormones in the present study. AUC of NPA of glucose was lower ($P < 0.05$) when pigs consumed the S4 diet than when they consumed the S1 diet at 0 to 4 h and the S2 diet at 4 to 10 h postprandial (**Figure 3.5A**).

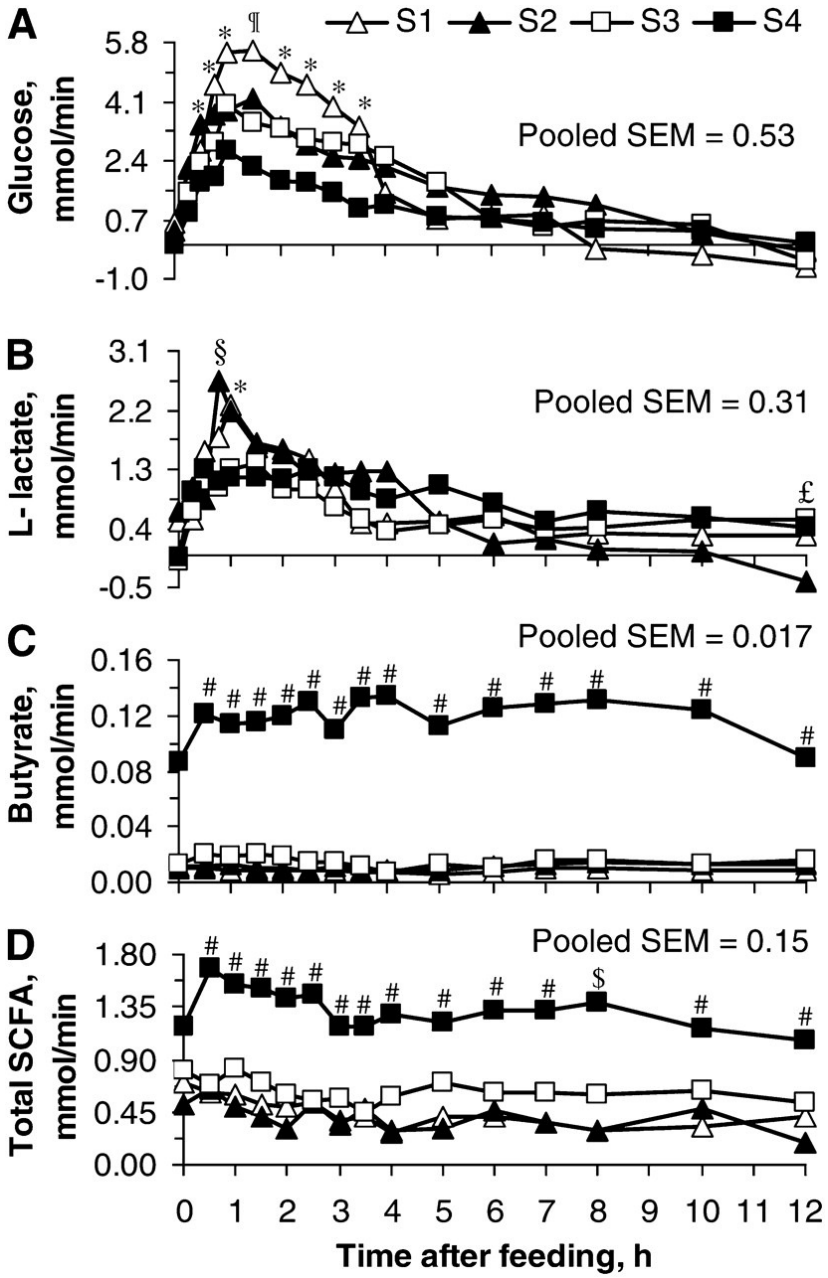

Figure 3.4 NPA of glucose (**A**), L-lactate (**B**), butyrate (**C**), and total SCFA (**D**) in pigs consuming starch diets differing in rate of digestion. Values are means, $n = 4$. Symbols indicate that means differ, $P < 0.05$: ¶, S1 > S2 = S3 > S4; *, S1 > S4; #, S1 = S2 = S3 > S4; §, S1 = S2 > S3 > S4.

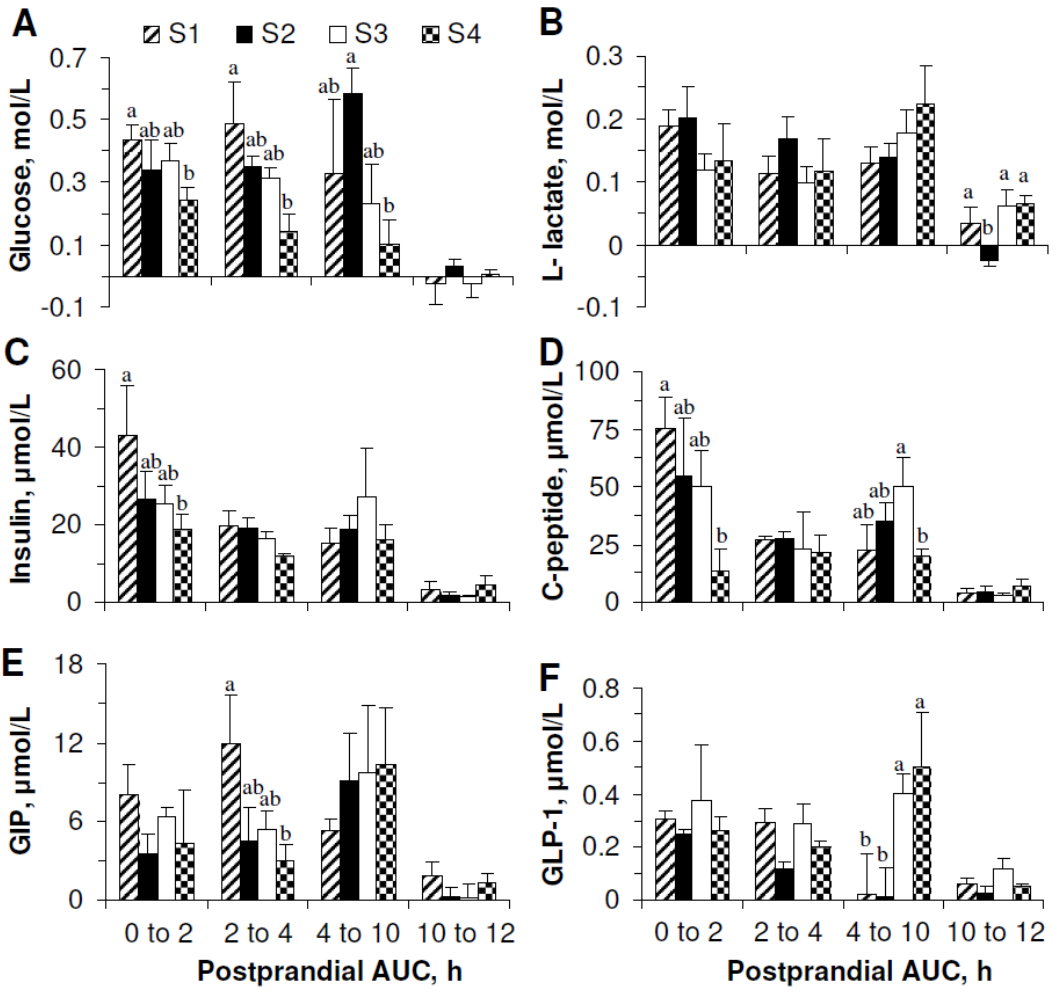

Figure 3.5 Postprandial areas under the curve of net portal appearance of glucose (**A**), L-lactate (**B**), insulin (**C**), C-peptide (**D**), GIP (**E**), and GLP-1 (**F**) during 0 to 2 h, 2 to 4 h; 4 to 10 h, and 10 to 12 h postprandial. Values are mean + SEM; n = 4. Labeled means without a common letter differ, $P < 0.05$.

3.3.2 Lactate and SCFA

When pigs consumed the S3 and S4 diets, NPA of L-lactate was lower ($P < 0.05$) than when they consumed the S1 diet at 1 h postprandial and when they consumed the S2 diet at 45 min and 1 h postprandial (**Figure 3.4B**). NPA of L-lactate was higher ($P < 0.05$) when pigs consumed the S4 diet than when they consumed the S1 and S2 diets at 12 h. AUC of NPA of L-lactate was lower ($P < 0.05$) when pigs consumed the S2 diet than when they consumed the other 3 diets at 10 to 12 h postprandial (**Figure 3.5B**). NPA of total SCFA from 30 min to 12 h and NPA of butyrate from 30 min to 10 h postprandial was higher ($P < 0.05$) when pigs consumed the S4 diet than when they consumed the other 3 diets (**Figure 3.4C** and **3.4D**). NPA of 12 h total SCFA as percentage of starch consumed was higher when pigs consumed the S4 diet than when they consumed the other 3 diets (**Table 3.5**). NPA of 12 h total SCFA tended to be higher ($P = 0.09$) when pigs consumed the S3 diet than when they consumed S1 and S2 diets. The molar proportion was lower ($P < 0.05$) for acetate, was higher ($P < 0.05$) for propionate, and tended to be higher ($P = 0.09$) for butyrate when pigs consumed the S4 diet than when they consumed the other 3 diets.

3.3.3 Insulin and C-peptide

When pigs consumed the S4 diet, the peak NPA of insulin and C-peptide was lower ($P < 0.05$) than when they consumed the S1 diet. The time required to peak and time required to halve the peak of insulin NPA did not differ ($P > 0.05$) among pigs consuming the four diets (**Table 3.3**). NPA of insulin was greater (*P*

< 0.05) when pigs consumed the S1 diet than when they consumed the S4 diet at 30 min and 1 h postprandial (**Figure 3.6A**). NPA of C-peptide was greater (P < 0.05) when pigs consumed the S1 diet than when they consumed the S4 diet at 30 min and 1.5 h; than when they consumed S2 and S3 diets at 1.5 h postprandial (**Figure 3.6B**). AUC of NPA of insulin and C-peptide was greater (P < 0.05) when pigs consumed the S1 diet than when they consumed the other 3 diets at 0 to 2 h postprandial (**Figure 3.5C** and **3.5D**). At 4-10 h postprandial, AUC of NPA of C-peptide was greater when pigs consumed the S2 diet than when they consumed the S4 diet. AUC of insulin did not differ (P > 0.05) among pigs consuming the four diets.

Table 3.5 NPA of major starch-derived nutrients in 12 h in pigs consuming 4 starch diets differing in rate of digestion[1]

	Starch diets				Pooled
Starch-derived nutrients	S1	S2	S3	S4	SEM
Total,[2] *% of starch consumed*	64.8	69.9	69.1	66.7	11.0
Glucose	49.7[a]	52.2[a]	49.6[a]	33.8[b]	8.6
L-lactate	9.17	11.1	10.6	12.3	2.44
Total SCFA	5.97[b]	6.59[b]	8.87[b]	20.5[a]	1.25
Acetate	5.46[c]	4.78[c]	7.66[b]	11.2[a]	0.738
Propionate	0.123[b]	1.37[b]	0.656[b]	5.65[a]	0.285
Butyrate	0.222[b]	0.186[b]	0.320[b]	2.51[a]	0.264
Molar proportions, *mol/100 mol*					
Acetate	89.4[a]	84.4[a]	86.8[a]	57.8[b]	3.13
Propionate	8.24[b]	12.2[b]	10.7[b]	29.5[a]	5.15
Butyrate	2.36	3.38	2.50	12.6	4.79

[1]Values are means and pooled SEM; n = 4; means in a row without a common superscript differ, P < 0.05. [2]The NPA of starch-derived nutrients was calculated as a percentage of starch consumed using the following assumptions: glucose in starch = NPA of (free) glucose × 0.9; 1 mol of glucose = 2 mol of L-lactate; NPA of SCFA from starch = total NPA of SCFA – NPA of SCFA from protein; NPA of SCFA from protei*n* = (isovalerate + isobutyrate) × 5; 1 mol of glucose = either 2 mol each of acetate, propionate, and isopropionate or either 1 mol each of butyrate, valarate, and caproate.

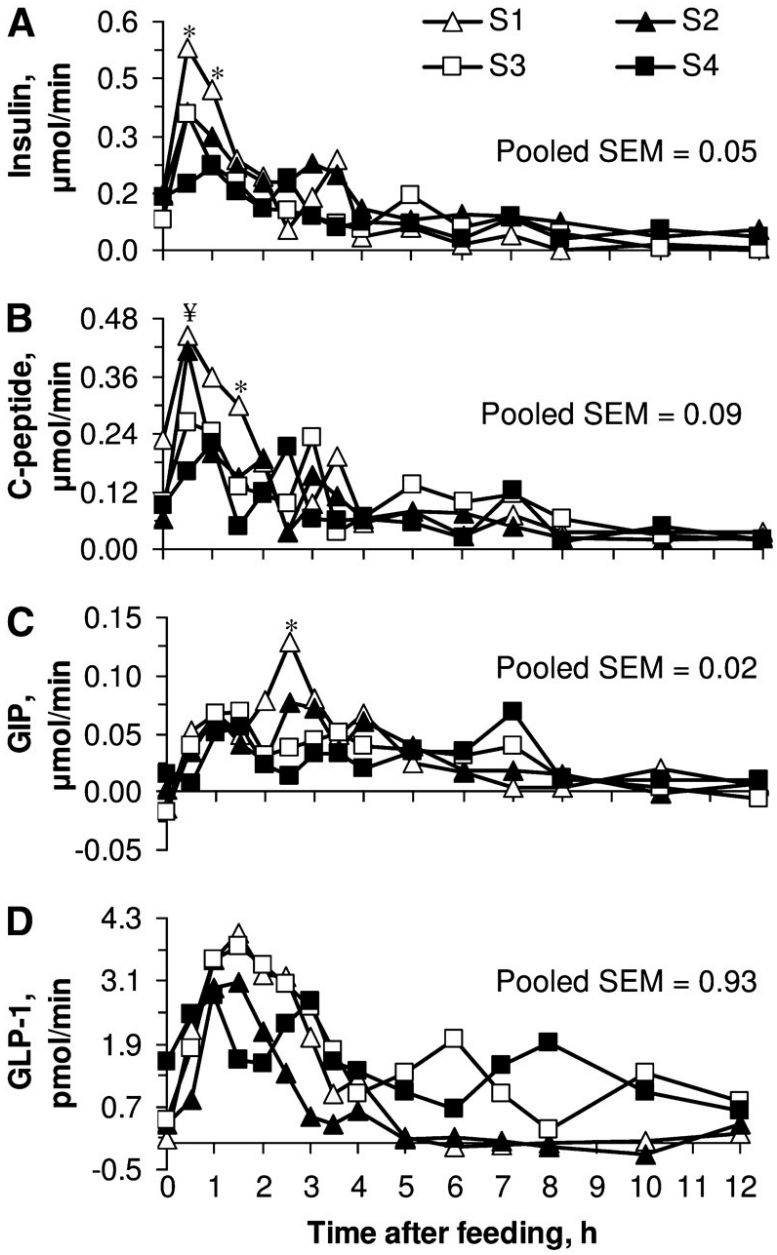

Figure 3.6 NPA of insulin (**A**), C-peptide (**B**), GIP (**C**), and GLP-1 (**D**) in pigs consuming starch diets differing in rate of digestion. Values are means, $n = 4$. Symbols indicate that means differ, $P < 0.05$: *, S1 > S4; ¥, S1 = S2 > S4.

3.3.4 Incretins

NPA of GIP was higher ($P < 0.05$) when pigs consumed the S1 diet than when they consumed the S4 diet at 2.5 h postprandial (**Figure 3.6C**). Although, NPA of GLP-1 did not differ ($P > 0.05$) among pigs consuming the four diets (**Figure 3.6D**), AUC of NPA of GLP-1 was higher ($P < 0.05$) when pigs consumed S3 and S4 diets than when they consumed S1 and S2 diets from 4 to 10 h postprandial (**Figure 3.5F**).

3.3.5 Relations of starch characteristics with NPA of nutrients, insulin, and incretins

Principal component analysis showed that PC 1 and PC 2 explained 79.2% of the total variability among starch characteristics and AUC of NPA of nutrients, insulin and incretins for different postprandial periods (**Figure 3.7**). Total in vitro digestion corrected for gastric emptying (van Kempen et al., 2010) had a strong positive relation [small angle among variables] with NPA of glucose, insulin, C-peptide and GIP and a strong negative relation [angle close to 180° among variables] with NPA of total SCFA during 0 to 2 and 2 to 4 h postprandial. In vitro digestion rate and NPA of glucose were positively associated at 0 to 2 and 4 to 10 h postprandial. Amylose had a strong negative relation with insulin during 0 to 2 h postprandial, weak relation during 2 to 4 h and 4 to 10 h postprandial. Crystallinity had a positive relation with NPA of glucose and insulin during 0 to 10 h postprandial (**Figure 3.7**).

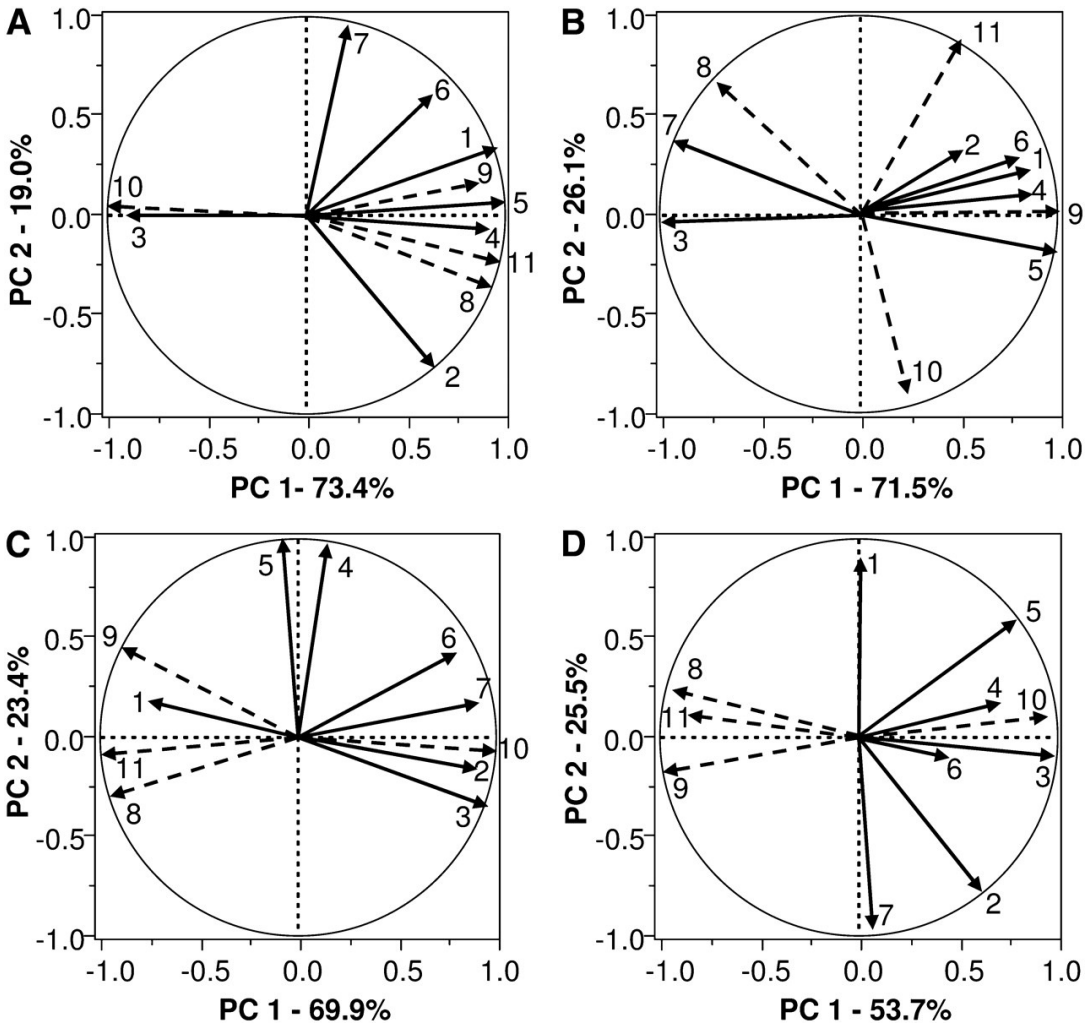

Figure 3.7 Loading plots of the first 2 eigenvalues (PC 1 and PC 2) showing the correlations among starch characteristics (dashed arrows) and AUC of the NPA of starch-derived nutrients and insulin and incretin secretion (solid arrows) at 0–2 h (**A**), 2–4 h (**B**), 4–10 h (**C**), and 10–12 h (**D**) postprandial in pigs consuming starch diets differing in rate of digestion; 1, glucose; 2, lactate; 3, total SCFA; 4, insulin; 5, C-peptide; 6, GIP; 7, GLP-1; 8, rate of in vitro digestion; 9, total in vitro digestion; 10, amylose; and 11, crystallinity. From 0 to 2 h; 2 to 4 h; 4 to 10 h; and 10 to 12 h postprandial, the NPA of glucose, respectively, increased and peaked, declined drastically, declined further toward zero, and was negligible; hence, the postprandial period may represent 4 distinct physiological phases.

3.3.6 Relations among nutrients and hormones

The kinetics of insulin secretion had a linear positive relation with kinetics of NPA of glucose (R^2 = 0.50; P < 0.01), GIP (R^2 = 0.42; P < 0.01), and GLP-1 (R^2 = 0.24; P < 0.01) (**Figure 3.8**). R^2 value from multiple regression of kinetics of NPA of glucose, L-lactate, total SCFA, GIP, GLP-1 with kinetics of NPA of insulin was not greater than that of kinetics of NPA of insulin with kinetics of NPA of glucose alone (data not shown). The R^2 of modeled NPA of insulin using a modified Chapman-Richards model and predicted NPA of insulin from modeled NPA of glucose was 0.55 (P < 0.01; **Figure 3.9**). When pigs consumed S1 and S2 diets, peak NPA of insulin was observed before peak NPA of glucose; while the peak NPA of insulin was observed after peak NPA of glucose when pigs consumed S3 and S4 diets (**Table 3.3; Figure 3.9; Figure 3.10**); in vitro starch digestion, therefore, predicted in vivo insulin release well. Kinetics of GIP secretion was related with kinetics of NPA of glucose (R^2 = 0.62; P < 0.01) and kinetics of GLP-1 secretion (R^2 = 0.43; P < 0.01). Kinetics of GLP-1 secretion was related with kinetics of NPA of glucose (R^2 = 0.47; P < 0.01) (**Figure 3.8**). Kinetics of insulin and C-peptide secretion were strongly related (R^2 = 0.80; P < 0.01). Similarly, the kinetics of NPA of glucose and L-lactate had a good relationship (R^2 = 0.67; P < 0.01) (**Figure 3.11**).

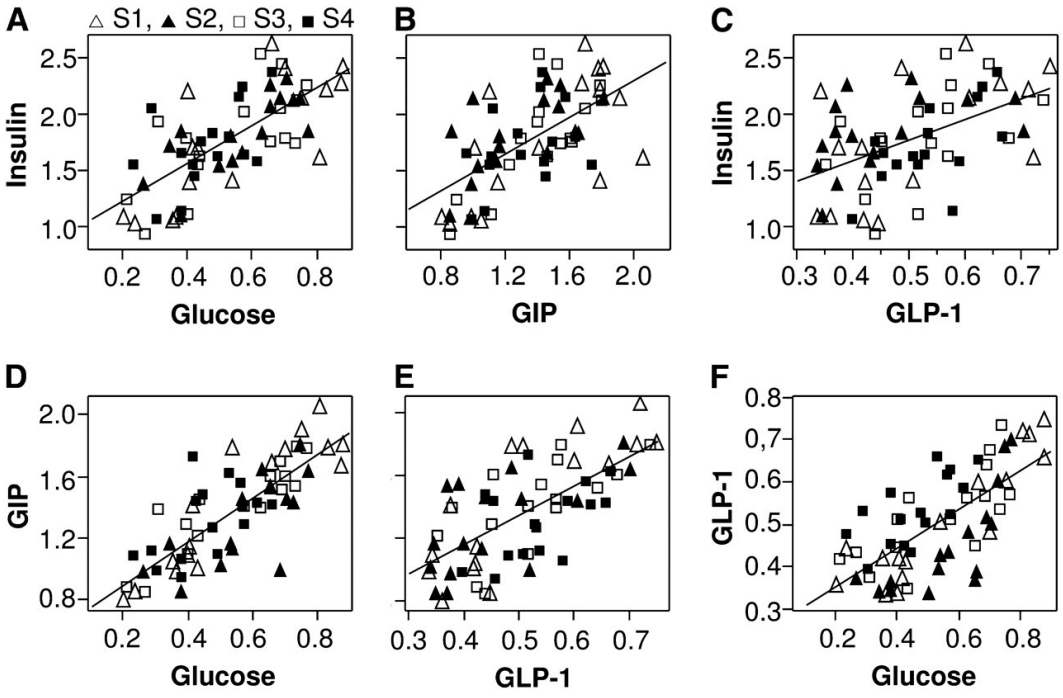

Figure 3.8 Relationships between NPA kinetics of insulin and glucose (R^2 = 0.50; root mean square error (RMSE) = 0.30; $P < 0.001$) (**A**), insulin and GIP (R^2 = 0.42; RMSE = 0.32; $P < 0.001$) (**B**), insulin and GLP-1 (R^2 = 0.24; RMSE = 0.37; $P < 0.001$) (**C**), GIP and glucose (R^2 = 0.62; RMSE = 0.19; $P < 0.001$) (**D**), GIP and GLP-1 total (r = 0.43; RMSE = 0.23; $P < 0.001$) (**E**), and GLP-1 and glucose (R^2 = 0.49; RMSE = 0.09; $P < 0.001$) (**F**) in pigs consuming starch diets differing in rate of digestion. Values are log-transformed means: insulin, GIP, and GLP-1 as log(pmol+2); glucose as log(mmol+2), n = 4.

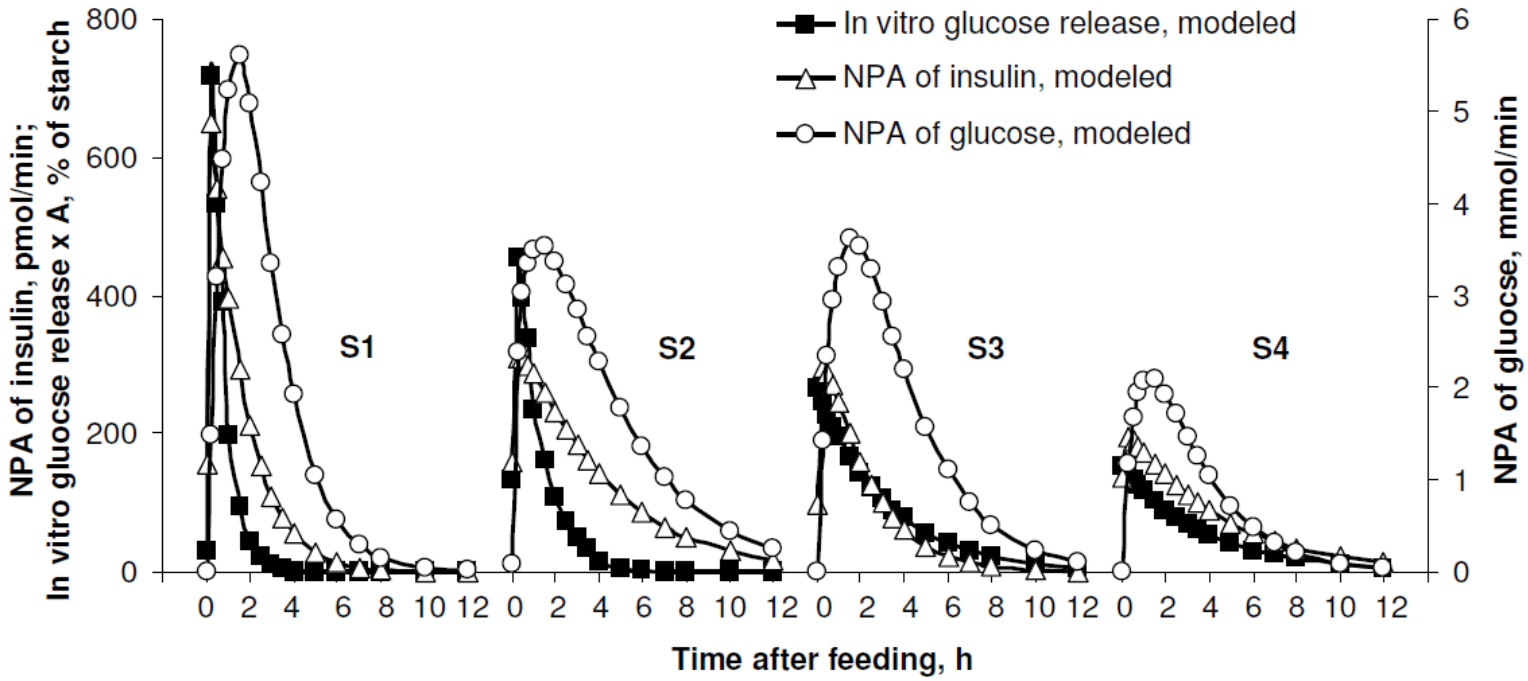

Figure 3.9 NPA of glucose and insulin in pigs consuming 4 starch diets differing in rate of digestion and in vitro glucose release corrected for gastric emptying; Chapman-Richards model (van Kempen et al., 2010) was used to get the model the variables from measured values (NPA of glucose, R^2 = 0.964; NPA of insulin, R^2 = 0.883); A, constant used to make the in vitro glucose release (% of starch) as the same scale as NPA of insulin (mmol/min). Values are predicted means; n = 4.

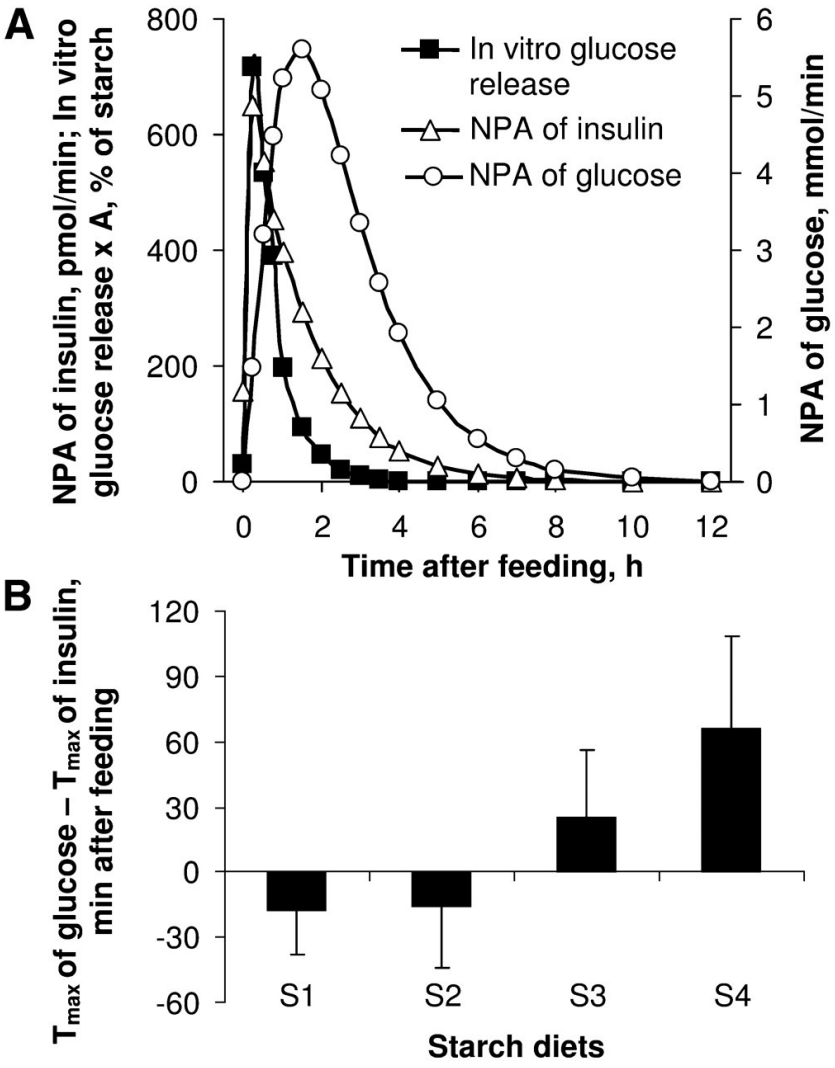

Figure 3.10 T_{max} for NPA of glucose and insulin in pigs consuming 4 starch diets differing in rate of digestion and peak in vitro glucose release.
(**A**) The NPA of glucose and insulin in pigs consuming the S1 diet and in vitro glucose release corrected for gastric emptying. A modified Chapman-Richards model (van Kempen et al., 2010) was used to model the variables from measured values (NPA of glucose, $R^2 = 0.97$; NPA of insulin, $R^2 = 0.87$). A constant was used to make the in vitro glucose release data (% of starch) on the same scale as NPA of glucose data (mmol/min). Values are predicted means, $n = 4$. (**B**) Difference in T_{max} of glucose and that of insulin was based on observed values; insulin peaked prior to glucose in pigs consuming S1 and S2 starch diets, whereas glucose peaked prior to insulin in pigs consuming S3 and S4 starch diets. Values are means and SEM, $n = 4$.

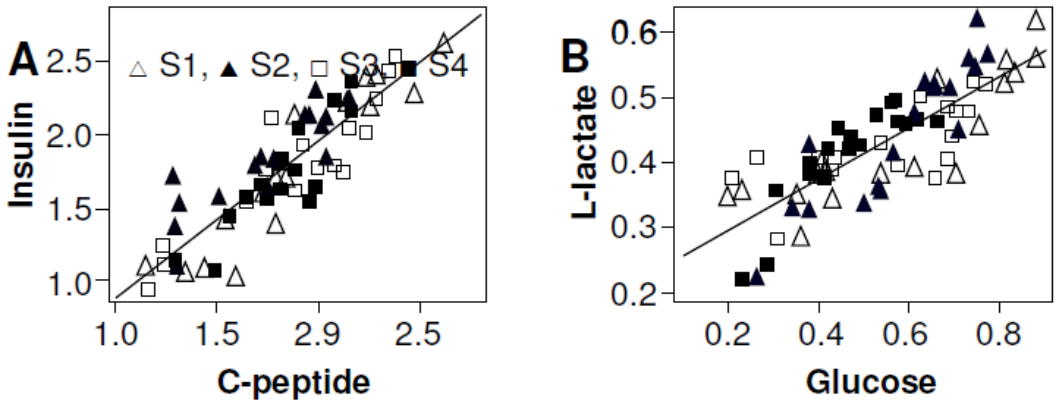

Figure 3.11 Relationship between NPA kinetics of insulin and C-peptide (R^2 = 0.80; RMSE = 0.20; $P < 0.001$; **A**), L-lactate and glucose (R^2 = 0.67; RMSE = 0.05; $P < 0.0001$; **B**) in pigs consuming starch diets differing in rate of digestion. Values are log transformed means: insulin and C-peptide as log(pmol+2); glucose and as log(mmol+2); n = 4.

3.4 Discussion

The present study evaluated effects of purified starches with a wide range in physicochemical properties using the portal vein-catheterized pig model. Pigs, having a similar digestive physiology and anatomy (Pond and Lei, 2001) and profile of carbohydrate-derived nutrients and pancreatic and gut hormones as humans (Spurlock and Gabler, 2008), can be used to understand the kinetics of nutrient absorption and pancreatic and gut hormone secretion. This model worked well as indicated by sustained catheters patency throughout the study, blood flow rate (Bach Knudsen et al., 2000; Hooda et al., 2009) and total portal glucose recovery (Rérat et al., 1984) similar to previous studies.

Generally, starches with high amylose and RS content are considered to reduce in vivo glucose and insulin responses; however, the in vivo response was not always consistent with pigs and humans (Noah et al., 2000; Higgins et al., 2004). Reasons

for the inconsistent response include: a) inconsistent and inadequate starch characterization; b) confounding effects of starch-associated compounds such as protein, fat, and fiber present in grains or diets; c) observation of glucose response in peripheral circulation as opposed to portal circulation; and d) coverage of limited postprandial duration (typically 120 min) to study in vivo responses. These issues were addressed using four approaches. First, starch sources were characterized in depth and in vitro starch digestion was used as a link between physicochemical properties of starch and in vivo responses (van Kempen et al., 2010). Second, purified starch instead of intrinsic starch was used in diets to avoid confounding effects of starch-associated compounds. Third, the NPA of starch-derived nutrients, insulin and incretins was used instead of their peripheral concentrations as a measure of in vivo starch effects so that net absorption of nutrients and secretion of insulin and incretins is properly measured and their hepatic metabolism is avoided. Fourth, in vivo responses were observed up to 12 h postprandial replacing typical 120 min in vivo response, because a major proportion of starch digestion can take place after 120 min postprandial (Muir and O'Dea, 1993; van Kempen et al., 2010).

3.4.1 Starch effect on glucose, insulin and C-peptide

Differences in postprandial glucose and insulin responses were detected when pigs (Deng et al., 2009) and human (Behall and Hallfrisch, 2002) consumed starches with a wide range in amylose and RS content. In the present study, greater peak glucose absorption was associated with greater secretion of insulin and C-peptide that resulted in a lower arterial plasma half-life of glucose.

Although peak glucose absorption was greater when pigs consumed S2 and S3 diets than when they consumed the S4 diet, peak insulin secretion did not differ when pigs consumed these 3 diets. This indicated that insulin responses do not exactly parallel glucose absorption or, more likely, the portal vein-catheterized pig model with 4 observations per treatment is not sensitive enough for detecting minor differences in hormone responses (Bach Knudsen et al., 2006).

Modeling indicated that secretion of insulin was delayed 5 to 15 min after glucose stimulation (Li et al., 2006). In contrast, modeled insulin secretion peaked prior to peak glucose absorption when pigs consumed S1 and S2 diets, and peaked after peak glucose absorption when pigs consumed S3 and S4 diets in the present study. Earlier peak insulin secretion when pigs consumed rapidly-digestible starch might be due to conditioned insulin secretion or stimulation of chemosensory proteins such as T1R sweet taste and G protein gustducin receptors in the tongue, stomach, and proximal small intestine (Breslin and Huang, 2006; Egan and Margolskee, 2008; Kokrashvili et al., 2009) in the presence of free glucose (Woods et al., 1977). In fact, stimulation of these receptors triggers entero-pancreatic neurons that cause pre-absorptive insulin secretion (Berthoud, 1984). The neural stimulation from cephalic and post-cephalic phases of food ingestion contributed up to 48% of total peripheral appearance of insulin (Berthoud, 1984).

3.4.2 Starch effects on lactate and SCFA

In the present study, total absorption of L-lactate was 9 to 12% of ingested starch, and slightly higher than the 7% of ingested starch measured in pigs consuming diets containing 57% starch (Noah et al., 2000). Glucose absorption and NPA of L-lactate were closely related. The conversion of glucose into L-lactate in the portal-drained viscera is thus proportional to glucose absorption during periods of high glucose availability in the gut. Previously, the starch effect on peripheral plasma lactate was inconsistent (Deng et al., 2009).

Total SCFA production in the gut, plasma concentration (van Munster et al., 1994), total absorption (Meijer et al., 1995; van der Meulen et al., 1997), and fecal butyrate concentration (Wang et al., 2002) was higher after feeding diets containing more RS or amylose in previous studies. In the present study, total SCFA and butyrate absorption was much greater when pigs consumed the S4 diet than when they consumed the other 3 diets, and did not differ when pigs consumed the S1, S2, and S3 diets. A major amount of RS is thus required to increase SCFA production and that low amounts of amylose can still be converted to glucose. Therefore, only the starch with very high amylose content and lower in vitro digestibility is available in major quantities for microbial fermentation.

When pigs consumed the S1 diet, 12 h glucose was higher and 12 h total SCFA absorption was lower than when they consumed the S4 diet, but 12 h NPA of total starch-derived nutrients did not differ when pigs consumed the four starch diets.

Thus, although much starch was not converted to glucose for the S4 diet, considerable undigested starch was fermented in the gut and absorbed as SCFA. Because energy production from SCFA is at least 14% lower than from glucose (Livesey, 1992; Jorgensen et al., 1997), high amylose starch is energetically less efficient than low amylose starch. Moreover, 30 - 35% of starch, which was not recovered in portal vein as glucose, lactate and SCFA, might have been partly used by PDV as energy, partly absorbed as alanine or intermediate metabolites of glycolysis and the Kreb's cycle and partly fermented in the large intestine beyond 12 h postprandial.

3.4.3 Starch effects on incretins

The cross-talk between intestine and pancreatic β-cells occurs partly via incretins, which play an important role in the regulation of blood glucose via mechanisms such as amplification of glucose-induced insulin secretion (Kellett et al., 2008). Incretin-stimulated insulin secretion accounts for at least 50% of insulin secreted after oral glucose consumption (Kim and Egan, 2008). Duodenal and proximal jejunal K cells release GIP, whereas distal jejunal, ileal and colonic L cells release GLP-1 (Nauck, 2009). Glucose in the lumen stimulates signal transduction via the apical membrane of K and L cells that facilitates incretin secretion (Raybould, 2007). Similar to the present study, peripheral GIP during the absorptive phase was positively correlated with plasma glucose in humans (Wachters-Hagedoorn et al., 2006). GLP-1 was, however, not affected by starches during 0 to 4 h postprandial; rather, it was greater when pigs consumed S3 and S4 diets than

when pigs consumed S1 and S2 diets during 4 to 10 h postprandial. Previously, peripheral GLP-1 in humans either increased (Raben et al., 1994; Juntunen et al., 2002) or unaffected (Wachters-Hagedoorn et al., 2006) during 0 to 2 h postprandial but decreased (Wachters-Hagedoorn et al., 2006) during 2 to 4 h postprandial after consuming diets with higher plasma glucose profile than that with lower plasma glucose profile. Glucose and SCFA act as potent stimulators of GLP-1 production (Drozdowski et al., 2002; Delzenne et al., 2005). Therefore, SCFA-induced GLP-1 production might have balanced the glucose induced GLP-1 production during 0 to 4 h postprandial and when pigs consumed S3 and S4 diets during 4 to 10 h postprandial in the present study.

3.4.4 Starch characteristics and metabolic responses

PC analysis revealed that the relationship of starch characteristics with the NPA of carbohydrate-derived nutrients, insulin, C-peptide, and incretins changed at different postprandial periods. Total in vitro digestion (corrected for gastric emptying) was strongly related with glucose, insulin, C-peptide, GIP, and total SCFA during 0 to 10 h postprandial and is thus a better indicator of total amount of starch available for enzymatic digestion and gut microbial fermentation than other starch characteristics. The current paradigm is that crystallinity reduces the rate of starch digestion (Singh et al., 2010); however, starch crystallinity was positively related with glucose absorption and insulin secretion during 0 to 4 h postprandial in the present study. Crystallinity effects of starch might have been confounded by effects of amylose content. Overall, our data indicate that in vitro

starch characteristics can better predict nutrient absorption and insulin and incretin secretion than starch physicochemical characteristics.

3.4.5 Relationships among nutrients and hormones

Digesta passage, glucose absorption, and SCFA production affect incretin secretion (Nauck, 2009), and blood glucose and incretin secretion affects insulin secretion (Ma et al., 2009). Peripheral insulin kinetics has been described using peripheral glucose kinetics using several models based on differential equations following oral and parenteral challenges with glucose and insulin (Makroglou et al., 2006). However, such models were not useful for the present study because, in contrast to glucose infusion or intake at specific time points, glucose was absorbed continuously following starch digestion.

In the present study, kinetics of insulin secretion was better related with kinetics of glucose absorption than other starch-derived nutrients. The kinetics of GIP and GLP-1 secretion and L-lactate absorption had a good relation with kinetics of insulin secretion. However, the addition of these 3 variables in the regression model did not improve the prediction of kinetics of insulin secretion beyond a prediction based solely on glucose absorption, indicating that the relation of glucose with L-lactate and incretins is not independent of the glucose and insulin relation. Glucose alone described half of the variation in NPA of insulin (R^2 = 0.55; $P < 0.01$). Thus, kinetics of glucose absorption and other factors such as

chemo-sensing in mouth or gut are major factors responsible for insulin secretion following intake of high dietary starch.

In conclusion, starch with high amylose content and lower rate and extent of in vitro digestion decreased glucose absorption and insulin secretion and increased SCFA absorption. Combining in vitro digestion and starch physicochemical characteristics describes in vivo starch effects well. Glucose absorption is an important modulator of insulin secretion; however, other physiological mechanisms such as chemo-sensing in the digestive tract must exist to explain the insulin response peaking prior to peak glucose uptake into the portal vein. Overall, the present study updated our current understanding of starch effects on the kinetics of nutrient absorption and insulin and incretin absorption and the information can be useful for the management of metabolic diseases and gut health in humans using functional foods.

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Chapter 4 Starch chemistry affects the kinetics of metabolites measured by NMR in portal and arterial circulation in pigs

4.1 Introduction

Starch types can influence profile of metabolites that are linked to occurrence of metabolic syndrome in human (Mathers and Daly, 1998) and to feed efficiency, meat quality and growth rate in swine (Weurding et al., 2001; van Kempen et al., 2007). Diets-influenced metabolites linked to such conditions in human and animals can be studied in plasma. Although numerous studies have been conducted to understand the starch effects on nutritional physiology, only limited numbers of metabolites were observed in plasma samples in these studies. Limitations in the total amount of blood samples that can be collected and limitations in traditional routine analytical approaches are among the major challenges for detailed metabolic profiling.

Nuclear magnetic resonance (NMR) can, both qualitatively and quantitatively, analyze metabolites from complex mixtures (Wishart, 2008). The advantages of NMR over other metabolomic analyses such as gas chromatography-mass spectrometry and traditional routine analytical approaches is that it is non-destructive, non-biased, easily quantifiable, requires little or no separation, permits the identification of novel compounds, and does not need chemical derivatization (Wishart, 2008). Due to such unique features, NMR has been increasingly used for metabolite profiling of body fluids such as plasma to understand the physiology with response to changes in environment including

dietary intake (Bertram et al., 2009; Lankinen et al., 2009). Recently, NMR has been started to be used in nutrition studies in monogastric species such as swine.

Starch containing high amylose and low in vitro digestibility decreased enzymatic starch digestion, glucose absorption (Chapter 1) and insulin secretion (Chapter 2) and increased starch fermentation and absorption in the gut (Chapter 2 and 4). In addition, such slowly-digestible starch modulated the secretion of incretins such as glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) (Chapter 2). The partial shift from starch digestion to starch fermentation can ultimately affect the profile of various metabolites present in plasma and NMR can be used to determine if such profile changes exist. In the present study, it was hypothesized that starch chemistry affects the kinetics of NMR-measured plasma metabolites such as carbohydrates, amino acids, organic acids, amines, and their derivatives. The objective was to determine the effect of starches with different rate of in vitro digestibility on NMR-measured plasma metabolites in pigs.

4.2 Materials and method

4.2.1 Starch sources and diet composition

Four purified starches widely differing in chemistry were used to formulate 4 high starch diets: a) Remyline AX-DR 0% amylose rice starch, b) Remy B7 20% amylose rice starch (Remy Industries, Leuven-Wijgmaal, Belgium), c) Nastar

35% amylose pea starch (Cosucra Group Warconing, Warconing, Belgium), and d) Gelose 80% amylose corn starch (Penford Food Ingredients, Centennial, CO) (Regmi et al., 2011). The starch diets were considered rapidly digestible (S1), moderately rapid digestible (S2), moderately slow digestible (S3), and slow digestible (S4) based on their rates of in vitro digestibility (1.92, 1.02, 0.38, and 0.22%/min, respectively) (Englyst et al., 2003).

4.2.2 Animal experiment

Animal Care and Use Committee for Livestock at the University of Alberta approved the animal use protocol of the study. Four female pigs (35.0 ± 0.2 kg initial BW) were catheterized in the portal vein and carotid artery (Hooda et al., 2009). After recovery, pigs were fed one of the four diets containing purified starches (**Table 2.2**) in a 4 × 4 Latin square design. Pigs were fed 1300 g/d in two equal meals at 0800 and 2000. The diets were formulated to meet the nutrients requirement of pigs based on NRC recommendations (NRC, 1998). On the d 7 of each period, blood samples from portal vein and carotid artery were collected 15 min before feeding and 1 h and 4 h after feeding. After centrifugation at 1500 × g for 10 min, plasma samples were stored at -80°C until further analysis.

4.2.3 NMR analysis

4.2.3.1 Sample preparation

All samples were filtered using 3 kDa molecular weight cut-off filters (Nanosep 3K Omega microcentrifuge filter tubes; Pall Corporation, NY, USA) to remove

macromolecules, including lipids and proteins. The filter tube manufacturer treats the filter membranes with glycerol as a preservative. The filters were washed 4-5 times with distilled water before use to remove the glycerol preservative. Samples that yielded less than 630 µL after filtration were diluted to 630 µL (300 mM KH_2PO_4 solution at pH 7) to ensure adequate volume for ^1H NMR acquisition. An 11.1% by volume internal standard solution (Chenomx Lot# 01-28-09-01, contains 4.5 mM DSS, 0.2 % w/v NaN_3, 10 mM difluorotrimethylsilanyl phosphonic acid in D_2O) was added to each sample solution that allows metabolite quantification, and the resulting mixture was vortexed for 30 s, resulting in a final volume of 700 µL. All metabolite concentrations obtained were adjusted by appropriate factors to account for the above dilutions, and represent the contents of the filtered samples, not the contents of the NMR tube. Mixed solution was then transferred to a 5 mm NMR tube (New Era Enterprises Inc., NJ, USA) for data acquisition.

4.2.3.2 Spectra acquisition and sample fitting

Spectra were acquired on a 600 MHz Varian INOVA spectrometer (Varian Inc., CA, USA) with 32 transients at 25ºC. Spectra were processed and CNX files were generated using the Processor module in Chenomx NMR Suite 6.0 software (Chenomx, Edmonton, Alberta, Canada). Spectra were zero filled to 64 k points and Fourier transformed. Spectral phasing was performed on the spectra along with baseline correction (Chang et al., 2007). Metabolites were identified and quantified with targeted profiling approach using the Profiler and Library

Manager modules in the same software, which contained 297 total metabolites and 54 typical plasma-measured metabolites. Minimum detection limit was approximately 2 μM. Five random spectra were used initially for a global search of all metabolites. Once a consensus metaprofile was created, the rest of the spectra were profiled using the consensus metaprofile. Additional features were searched for and added on an individual spectrum basis. Each spectrum was peer reviewed by a separate analyst. A final review pass was done on all spectra before exporting concentration results. Concentration measurements were adjusted to report metabolite concentrations after filtration of the plasma.

4.2.3.3 Metabolites analysis by traditional analysis

Plasma samples were analyzed for glucose using glucose oxidase kits (Diagnostics Chemicals Ltd., Charlottetown, Prince Edward Island, Canada) and lactate using lactate dehydrogenase kit (R-Biopharm, Darmstadt, Germany). Acetate was analyzed by gas chromatography (Hewlett Packard, Waldbroom, Germany) using a method described previously (Brighenti, 1997) with some modifications. Briefly, the plasma samples were deproteinized using 25% phosphoric acid solution (8 μL/100 μL of plasma), after adding isocaproic acid as an internal standard, SCFA were analyzed in split-less mode and using He as a carrier gas, 30 m × 0.53 mm × 1 μm size column, and using an FID detector.

4.2.4 Statistical analysis

Starch effects on metabolites were analyzed using a mixed model with diet as a fixed effect and period and diets as a random effect in JMP software (version 8.0.2; SAS Inst. Inc., Cary, NC). All variables were tested for normal distribution by the Kolmogoroff-Smirnoff test. Pigs were considered the experimental units and significance of difference was set at $P < 0.05$. Values reported are means ± pooled SEM. Regression and multivariate analysis was conducted to determine the relationship among variables and starches. Data were log transformed to meet the requirement of regression analysis. To reflect the differences and similarity between the starch diets, partial least squares-discriminant analysis (PLS-DA) models were constructed using SIMCA-P+ software (version 12.0.1; Umetrics AB, Umeå, Sweden). A validation plot using 20 permutations was used to check the validity and the degree of overfit for the PLS model. Potential biomarkers affecting the PLS-DA scatter plot were identified using Variable Importance in the Projection (VIP) plot. Principal component (PC) analysis based on correlation of metabolites was conducted using JMP software (version 8.0.2; SAS Inst. Inc., Cary, NC). The length and the angles between the straight lines were used to describe the inter-relationship among the variables.

4.3 Results

Out of 297 total metabolites and 54 typical plasma-measured metabolites present in the Profiler and Library Manager modules of Chenomx NMR Suite 6.0 software, 28 metabolites were identified and quantified in the present study

(**Figure 4.1**). The metabolites groups include amides, amines, amino acid derivatives, amino acids, ammonium compounds, aromatic compounds, fatty acids, nucleic acid components, organic acids and sugars. Among the metabolites identified, 2-hydroxybutyrate, hypoxanthine, and pyruvate were quantified in less than 80% of the samples (**Table 4.1**).

Score plots from PLS-DA showed that S4 starch diet had distinctly different overall profile of NMR-measured metabolites before feeding, as well as 1 and 4 h postprandial in carotid and portal plasma compared to other starch diets (**Figure 4.2 and 4.3**). Variable influence in the projection (VIP) plots showed that acetate and betaine were among the 4 most influencing biomarkers for PLS-DA in carotid plasma and before feeding in portal plasma; whereas glucose, lactate, acetate and alanine were the top 4 biomarkers influencing the PLS-DA at 1 h postprandial and succinate, glutamate, formate and betaine were the top 4 biomarkers influencing the PLS-DA at 4 h postprandial in portal plasma.

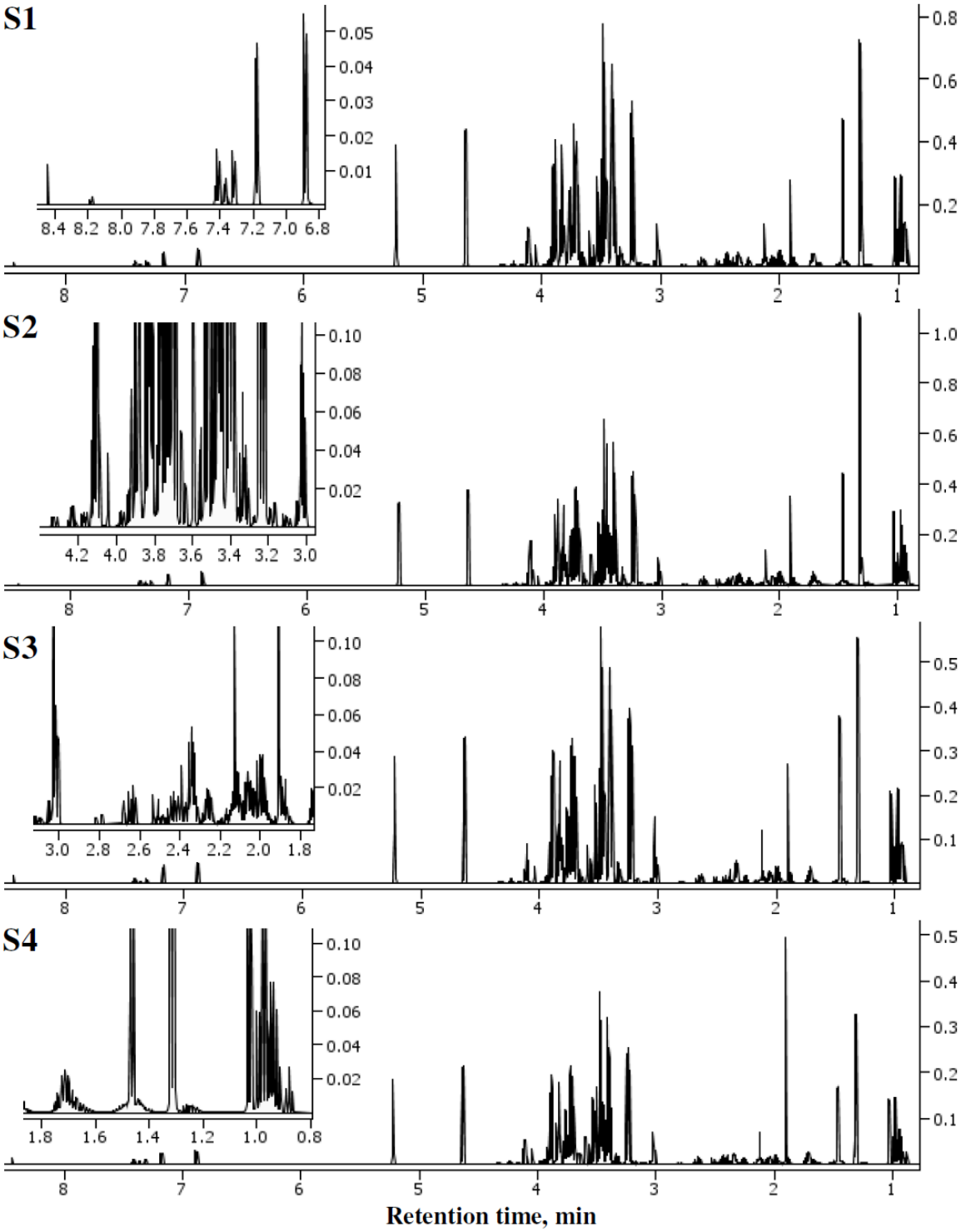

Figure 4.1 Representative NMR spectra of 28 metabolites from plasma of pigs fed S1, S2, S3, and S4 starch diets; compound identification and respective peaks are given in Table 2.

Table 4.1 Concentrations of NMR metabolites in plasma samples of pigs consuming 4 starch diets differing in rate of digestion.

Compound	Average, µMol	Min, µMol	Max, µMol	Occurrence, %	Peak observed in the NMR, min
Carbohydrates and Derivatives					
Glucose	9755	3857	24238	100	5.2, 4.6, 3.9, 3.8, 3.8, 3.8, 3.7, 3.7, 3.5, 3.5, 3.5, 3.4, 3.4, 3.2
Glycerol	202	39	775	100	3.8, 3.6, 3.6
Amines and derivatives					
Creatine	199	24	688	100	3.9, 3.0
Creatinine	165	63	328	100	4.0, 3.0
Organic acids					
Acetate	454	78	1679	100	1.9
Citrate	141	44	254	100	2.7, 2.5
Formate	55	14	141	96	8.4
Lactate	2006	670	5324	100	4.1, 1.3
Succinate	11	3	22	93	2.4
2-Hydroxybutyrate*	53	9	194	31	4.0, 1.7, 1.6, 0.9
Hypoxanthine*	23	12	44	68	8.2, 8.2
Pyruvate*	48	7	140	68	2.4
Amino Acids and Derivatives					
Alanine	1185	379	2347	100	3.8, 1.5
Glutamate	587	203	1293	100	3.7, 2.4, 2.3, 2.1, 2.0
Glutamine	420	63	1015	99	7.6, 6.9, 3.8, 2.5, 2.4, 2.1, 2.1
Isoleucine	355	96	816	100	3.7, 2.0, 1.4, 1.3, 1.0, 0.9
Leucine	431	110	999	100	3.7, 1.7, 1.7, 1.7, 1.0, 0.9
Lysine	470	102	970	100	3.7, 3.0, 1.9, 1.9, 1.7, 1.5, 1.4
Methionine	142	25	335	100	3.8, 2.6, 2.2, 2.1, 2.1
Phenylalanine	137	27	307	99	7.4, 7.4, 7.3, 4.0, 3.3 ,3.1
Proline	820	162	1721	100	4.1, 3.4, 3.3 ,2.3, 2.1, 2.0, 2.0
Threonine	386	57	877	100	4.2, 3.6, 1.3
Tyrosine	369	80	781	100	7.2, 6.9, 3.9, 3.2, 3.0
Valine	892	328	1629	100	3.6, 2.3, 1.0, 1.0
Aspartate	91	17	174	85	3.9, 2.8, 2.7
Betaine	62	12	143	100	3.9, 3.3
Pyroglutamate	138	49	246	100	7.7, 4.2, 2.5, 2.4, 2.4, 2.0
trans-4-Hydroxy-L-proline	177	68	329	96	4.7, 4.3, 3.5, 3.4, 2.4, 2.1

*Metabolites with less than 80% occurrence were not used for further analysis

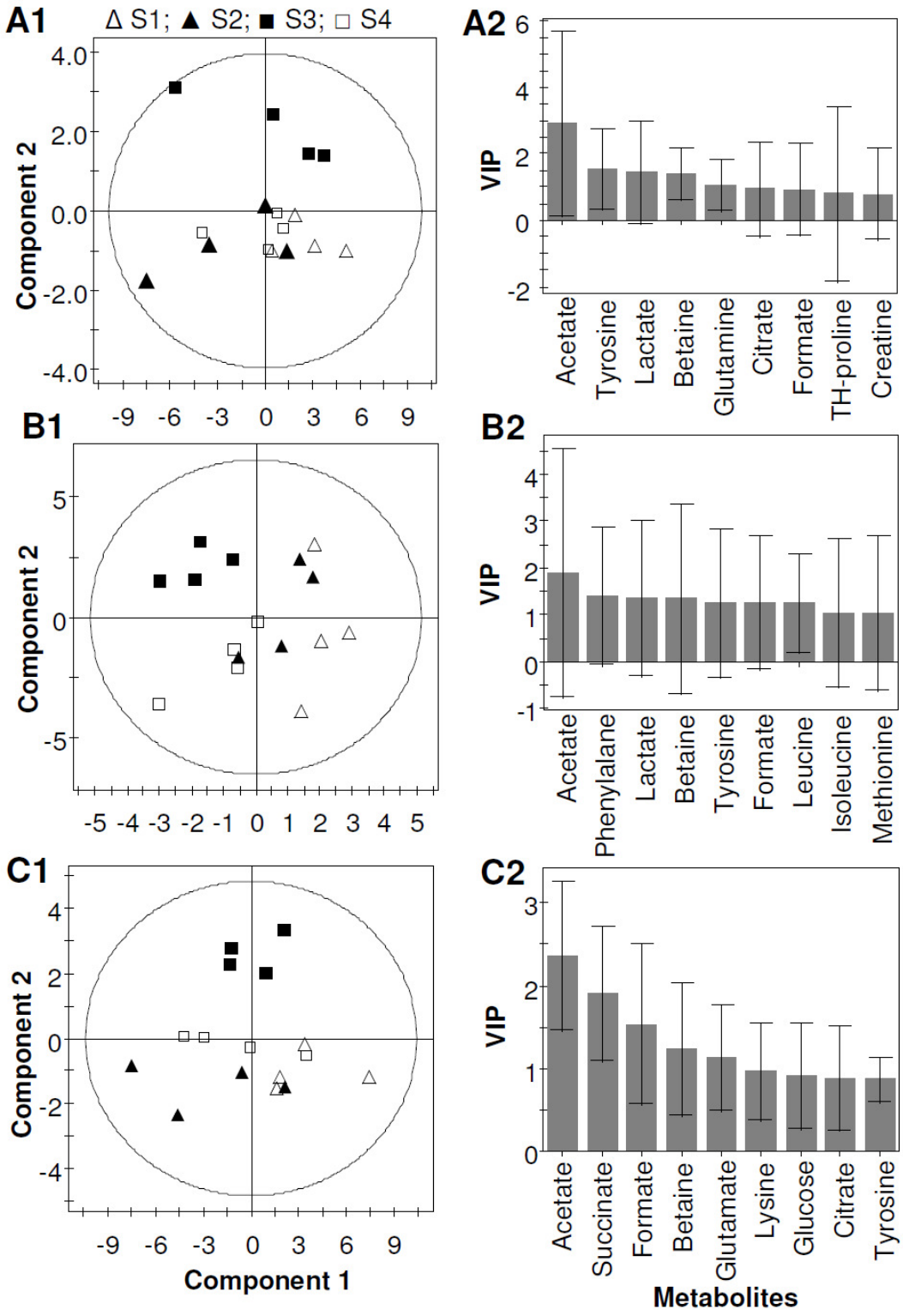

Figure 4.2 PLS-DA of NMR metabolites in carotid plasma of pigs consuming 4 starch diets differing in rate of digestion; A1, before feeding; B1, 1 h postprandial; C1, 4 h postprandial; A2, B2 and C2, respective VIP plots.

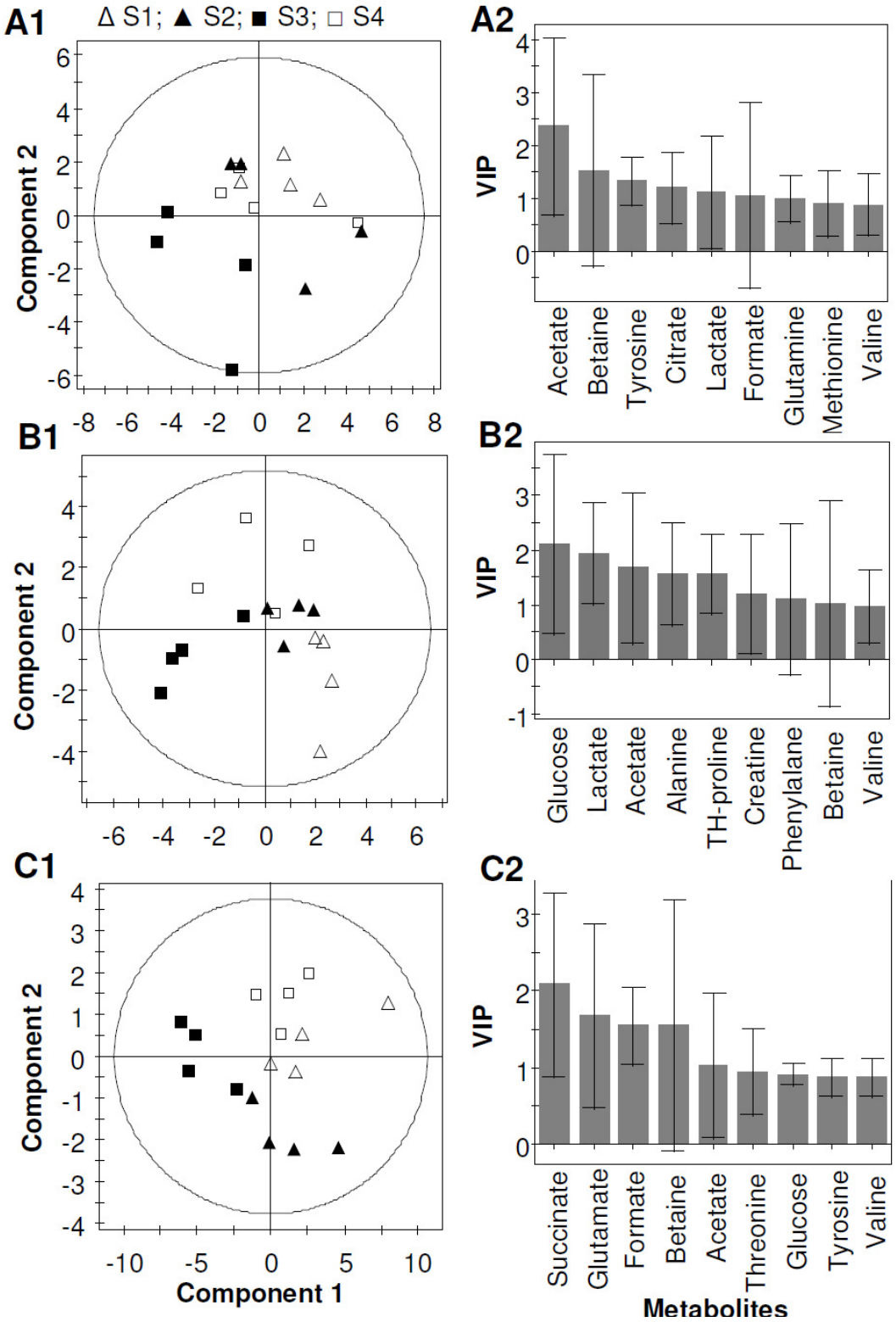

Figure 4.3 PLS-DA of NMR metabolites in portal plasma of pigs consuming 4 starch diets differing in rate of digestion; A1, before feeding; B1, 1 h postprandial; C1, 4 h postprandial; A2, B2 and C2, respective VIP plots.

The starch diet effects on the biomarkers with greater influence on PLS-DA plots as indicated by VIP plots were analyzed by mixed model procedure. The analysis revealed statistically significant effect of starch diets on different biomarkers. When pigs were fed the S4 diet, acetate concentration increased ($P < 0.05$) at all time points in both carotid and portal plasma; formate concentration increased ($P < 0.05$) in carotid plasma 1 h after feeding; succinate concentration increased ($P < 0.05$) in carotid plasma 4 h after feeding; betaine concentration increased ($P < 0.05$) in portal plasma before feeding and 1 h after feeding; glucose concentration decreased ($P < 0.05$) in portal plasma at 1 and 4 h after feeding; lactate concentration decreased ($P < 0.05$) 1 h after feeding; threonine, tyrosine and valine in portal plasma decreased ($P < 0.05$) at 4 h after feeding (**Table 4.2 and 4.3**).

Table 4.2 Concentration of NMR-measured metabolites carotid plasma of pigs consuming 4 starch diets differing in rate of digestion[1].

Metabolites	Starch diets				Pooled SEM	*P* Value
	S1	S2	S3	S4		
Before feeding						
Acetate, *mmol/L*	0.13[b]	0.20[ab]	0.20[ab]	0.52[a]	0.032	0.01
Betaine, *μmol/L*	20.1	70.4	39.5	64.5	16.37	0.20
Formate, *μmol/L*	15.1	22.0	28.2	29.1	6.57	0.27
Glutamine, *mmol/L*	0.13	0.23	0.15	0.26	0.095	0.44
Lactate, *mmol/L*	1.00	1.76	1.39	1.01	0.182	0.96
Citrate, *μmol/L*	67.6	97.1	98.2	102.6	12.67	0.19
Creatine, *μmol/L*	89.6	216.6	63.7	64.8	109.13	0.89
Tyrosine, *mmol/L*	0.15	0.24	0.19	0.14	0.042	0.82
trans-4-hydroxy-L-proline, *mmol/L*	0.11	0.13	0.16	0.10	0.008	0.66
Glucose, *mmol/L*	5.04	6.81	6.09	6.00	0.804	0.10
1 h after feeding						
Acetate, *mmol/L*	0.22[b]	0.21[b]	0.28[ab]	0.60[a]	0.055	0.04
Phenylalanine, *mmol/L*	0.15	0.16	0.22	0.19	0.019	0.28
Lactate, *mmol/L*	3.80	2.98	2.54	2.30	0.436	0.15
Betaine, *μmol/L*	44.4	87.1	59.8	95.7	15.97	0.15
Tyrosine, *mmol/L*	0.47	0.50	0.61	0.40	0.055	0.50
Formate, *μmol/L*	38.1	36.0	31.6	61.2	3.98	0.05
Leucine, mol	0.56	0.55	0.73	0.67	0.073	0.41
Isoleucine, mol	0.49	0.47	0.59	0.50	0.025	0.80
Methionine, mol	0.18	0.18	0.24	0.18	0.018	0.93
Glucose, *mmol/L*	11.7	9.4	10.6	10.1	0.45	0.10
4 h after feeding						
Acetate, *mmol/L*	0.11[b]	0.18[ab]	0.20[ab]	0.52[a]	0.016	0.00
Succinate, *μmol/L*	6.03[b]	3.81[b]	8.89[ab]	12.39[a]	0.73	0.03
Formate, *μmol/L*	37.5	42.0	76.9	83.3	11.89	0.11
Betaine, *μmol/L*	29.8	96.8	54.6	102.5	13.68	0.06
Glutamate, *mmol/L*	0.37	0.58	0.73	0.69	0.093	0.13
Lysine, *mmol/L*	0.38	0.58	0.52	0.38	0.092	0.98
Glucose, *mmol/L*	6.20	9.38	9.15	9.55	1.792	0.32
Citrate, *mmol/L*	0.12	0.17	0.14	0.12	0.019	0.81
Tyrosine, *mmol/L*	0.28	0.47	0.42	0.34	0.059	0.57
Lactate, *mmol/L*	1.00	1.62	1.51	1.50	0.068	0.35

[1]Values are means and pooled SEM, n = 4, means in a row with superscripts without a common letter differ.

Table 4.3 Concentration of NMR-measured metabolites portal plasma of pigs consuming 4 starch diets differing in rate of digestion[1].

Metabolites	Starch diets				Pooled SEM	*P* Value
	S1	S2	S3	S4		
Before feeding						
Acetate, *mmol/L*	0.44[b]	0.63[ab]	0.61[ab]	1.12[a]	0.079	0.03
Betaine, *μmol/L*	34.9[b]	50.2[ab]	39.2[ab]	64.8[b]	3.85	0.03
Tyrosine, *mmol/L*	0.23	0.25	0.21	0.14	0.031	0.19
Citrate, *mmol/L*	0.11	0.13	0.11	0.11	0.027	0.92
Lactate, *mmol/L*	1.54	1.67	1.61	1.04	0.290	0.35
Formate, *μmol/L*	34.7	29.8	36.0	51.1	6.05	0.19
Glutamine, *mmol/L*	0.21	0.25	0.24	0.19	0.105	0.89
Methionine, *μmol/L*	67.0	65.5	56.9	47.6	14.22	0.44
Valine, *mmol/L*	0.60	0.62	0.52	0.48	0.088	0.44
Glucose, *mmol/L*	7.07	6.66	6.68	5.88	0.745	0.10
1 h after feeding						
Glucose, *mmol/L*	19.7[a]	14.7[ab]	12.7[b]	12.0[b]	1.86	0.05
Lactate, *mmol/L*	4.18[a]	3.52[ab]	2.55[ab]	2.43[b]	0.244	0.04
Acetate, *mmol/L*	0.40[b]	0.48[ab]	0.46[ab]	1.12[a]	0.027	0.00
Alanine, *mmol/L*	1.93[a]	1.92[a]	1.83[ab]	1.48[b]	0.122	0.05
trans-4-hydroxy-L-proline, *mmol/L*	0.23	0.22	0.20	0.15	0.023	0.14
Creatine, *mmol/L*	0.26	0.29	0.20	0.16	0.047	0.27
Phenylalanine, *mmol/L*	0.20	0.22	0.25	0.24	0.018	0.28
Betaine, *μmol/L*	43.3[b]	68.1[ab]	47.6[ab]	78.6[a]	5.90	0.05
Valine, *mmol/L*	1.27	1.23	1.16	1.09	0.111	0.36
4 h after feeding						
Succinate, *μmol/L*	14.7	10.2	16.6	12.0	1.64	0.36
Glutamate, *mmol/L*	0.56	0.42	0.70	0.40	0.082	0.31
Formate, *μmol/L*	97.6	70.4	114.6	96.1	8.87	0.91
Betaine, *μmol/L*	46.4	81.1	55.5	74.2	6.27	0.09
Acetate, *mmol/L*	0.44[b]	0.39[ab]	0.55[ab]	0.99[a]	0.045	0.01
Threonine, *mmol/L*	0.48[a]	0.47[a]	0.42[ab]	0.27[b]	0.007	0.00
Glucose, *mmol/L*	15.1[a]	12.7[ab]	13.2[ab]	7.8[b]	1.60	0.05
Tyrosine, *mmol/L*	0.49[a]	0.49[a]	0.50[ab]	0.27[b]	0.027	0.03
Valine, *mmol/L*	1.29[a]	1.08[ab]	1.01[ab]	0.6[b]	0.114	0.07
Lactate, *mmol/L*	2.06	2.03	1.89	1.22	0.355	0.08

[1]Values are means and pooled SEM, $n = 4$, means in a row with superscripts without a common letter differ.

In addition to NMR analysis, glucose, lactate and acetate were determined by routine analyses. The analysis showed that lactate concentration and glucose concentration before feeding in both portal and carotid plasma was not affected when pigs were fed different starch diets. When pigs were fed the S4 diet, acetate concentration was increased before feeding and 4 h after feeding in carotid plasma, increased 1 and 4 h after feeding in portal plasma and glucose concentration was decreased 1 and 4 h after feeding in both carotid and portal plasma (**Table 4.4**). For, glucose, lactate and acetate NMR-measured data was positively correlated ($P < 0.01$) with the data obtained from routine analysis (**Figure 4.4**).

Loading plots from PC Analysis showed the interrelationship among the metabolites in carotid and portal plasma (**Figure 4.5**). There were 2 distinct clusters of metabolites: a) betaine and acetate and formate group and b) glucose, lactate, amines and amino acids group. In portal plasma these 2 groups were consistently present in different quadrant in the loading plots indicating that these two groups were differentially affected by PC1 and 2. In carotid plasma, acetate and formate had consistently lower angle with betaine compared to other metabolites. In addition, glucose and lactate tended to remain closely associated at 1 and 4 h postprandial in both carotid and portal plasma; and amines and essential and non essential amino acids tended to remain closely associated and with glucose and lactate.

Table 4.4 Concentration of glucose, acetate, and lactate determined by routine analytical procedure in plasma of pigs consuming 4 starch diets differing in rate of digestion[1].

Metabolites	Starch diets				Pooled	*P*
	S1	S2	S3	S4	SEM	value
Carotid before feeding						
Glucose, *mmol/L*	4.53	5.02	5.10	5.47	0.351	0.635
Lactate, *mmol/L*	1.89	2.13	1.25	1.81	0.364	0.419
Acetate, *mmol/L*	0.25	0.13	0.22	0.24	0.038	0.182
Carotid 1 h after feeding						
Glucose, *mmol/L*	6.51^{a}	6.26^{a}	5.37^{ab}	4.80^{b}	0.545	0.054
Lactate, *mmol/L*	3.90	3.81	2.92	3.22	0.730	0.744
Acetate, *mmol/L*	0.20	0.24	0.19	0.20	0.060	0.913
Carotid 4 h after feeding						
Glucose, *mmol/L*	5.26	5.38	5.76	5.50	0.391	0.83
Lactate, *mmol/L*	2.10	1.81	2.58	2.45	0.286	0.29
Acetate, *mmol/L*	0.22^{b}	0.24^{b}	0.21^{b}	0.42^{a}	0.029	0.02
Portal before feeding						
Glucose, *mmol/L*	4.93	5.42	4.63	5.08	0.34	0.236
Lactate, *mmol/L*	1.91	2.35	1.64	1.56	0.423	0.576
Acetate, *mmol/L*	0.97	0.80	0.85	0.86	0.215	0.946
Portal 1 h after feeding						
Glucose, *mmol/L*	12.12^{a}	10.04^{a}	10.19^{ab}	8.83^{b}	0.77	0.05
Lactate, *mmol/L*	6.13	6.09	4.26	4.28	0.829	0.257
Acetate, *mmol/L*	0.66^{b}	0.63^{b}	0.84^{ab}	1.16^{a}	0.112	0.038
Portal 4 h after feeding						
Glucose, *mmol/L*	7.05^{ab}	8.93^{a}	8.95^{a}	6.65^{b}	0.31	0.05
Lactate, *mmol/L*	2.11	2.91	3.51	2.71	0.69	0.57
Acetate, *mmol/L*	0.46^{b}	0.50^{b}	0.77^{ab}	0.95^{a}	0.07	0.01

[1]Values are means and pooled SEM, n = 4, means in a row with superscripts without a common letter differ.

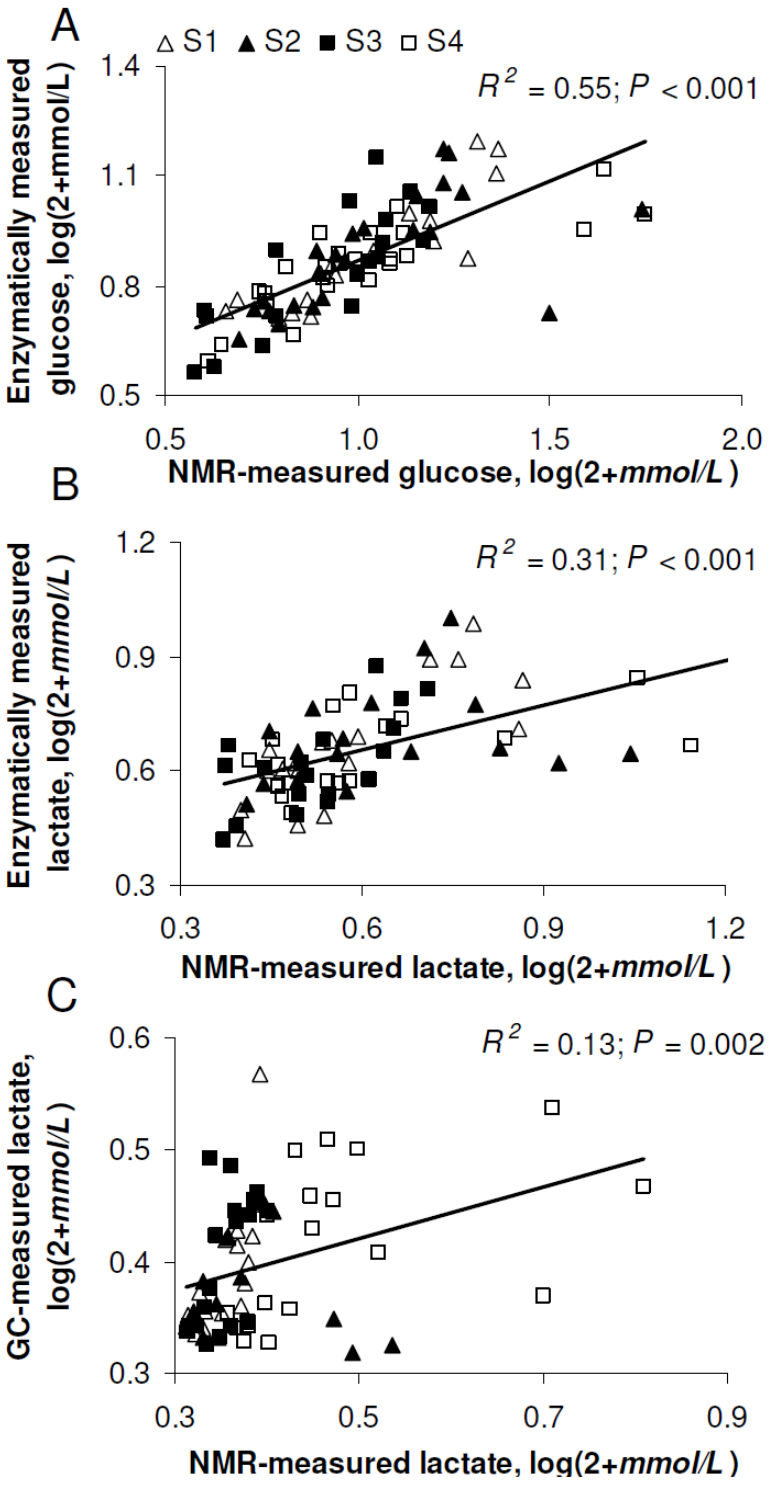

Figure 4.4 Regression of traditionally measured vs. NMR-measured results for glucose (A), lactate (B), and acetate (C).

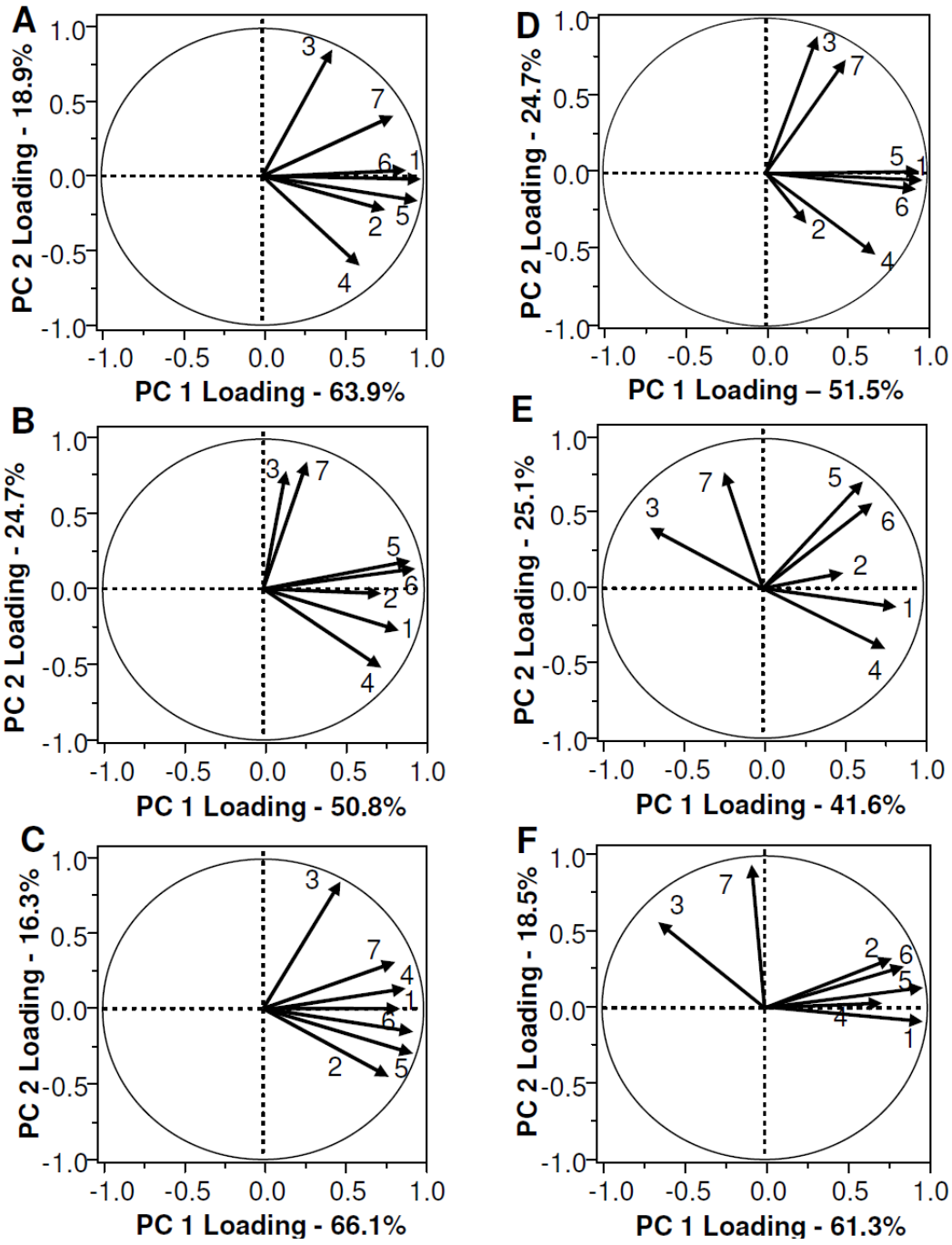

Figure 4.5 Interrelationship among plasma metabolites in pigs fed starch diets as determined by principal component analysis; A, carotid before feeding; B, carotid 1 h postprandial; C, carotid 4 h postprandial; D, portal before feeding; E, portal 1 h postprandial; F, portal 4 h postprandial; 1, glucose; 2, amines; 3, acetate and formate; 4, lactate; 5, essential amino acids; 6, non essential amino acids; 7, betaine.

4.4 Discussion

Because of high throughput nature and having exceptional capacity to handle complex metabolite mixtures, NMR is emerging as a preferred technology for the analysis of metabolites in research studies including effect of dietary interventions such as carbohydrate on serum metabolic profile in recent years (Wishart, 2008; Lankinen et al., 2009). NMR has also overcome the problem of low sample quantity which has been a major limitation of metabolic profiling studies and kinetics studies which often involves repeated collection of sample such as blood over time from the same experimental subject. In the present study, NMR technology was used for metabolic profiling of portal and carotid plasma samples from a nutrient kinetics study in pigs, effect of different starch diets on these metabolites studied, and some of the NMR results were compared with results from routine analytical procedure. Portal metabolites represent the nutrients absorption from intestine and metabolism in portal drained viscera (PDV); whereas carotid metabolites represent the overall profile of nutrients, intermediate metabolic products and excretory products.

Out of total 54 metabolites that are present in the database library of Chenomx NMR Suite 6.0 software and normally detected by NMR in plasma, 28 metabolites were identified in the present study. Remaining 26 metabolites present in the database library were below the detection limit of NMR, 2 *μmol/L*. Out of the 28 detected metabolites, 23 were quantified in all of the samples. Other

metabolites that are likely present in pig plasma were not detected due to the limitation of database library of Chenomx NMR Suite 6.0 software.

NMR-measured value had positive linear correlation ($P < 0.01$) with traditionally measured value for glucose ($R^2 = 0.55$), lactate ($R^2 = 0.31$) and acetate ($R^2 = 0.13$) and the overall trend of starch diet effects on glucose, lactate and acetate in portal and carotid plasma determined by routine analytical procedure were similar to that quantified by NMR. This result, in general, validated the use of NMR analysis to understand the effect of dietary treatments on the overall profile of plasma metabolites in pigs.

Starch diet effects on overall profile of metabolites were evident in the plasma samples as revealed by PLS-DA score plots. As shown by the VIP plots, acetate, betaine, formate, lactate, glucose, tyrosine, phenylalanine, alanine, glutamate, succinate, and citrate are among the top 4 metabolites influencing the starch dissimilarity seen in PLS-DA plots. Acetate is produced from microbial fermentation of carbohydrates and formate is an intermediate metabolite of acetate production (Flint et al., 2008). As more carbohydrate is available for microbial fermentation, more of these compounds are expected to be produced in the gut and eventually absorbed in the portal circulation. Glucose is absorbed from the gut after enzymatic digestion of starch. Lactate is produced in the body from pyruvate, the product of glycolysis. Succinate and citrate are intermediary metabolites of the Kreb's cycle. Amino acids are present in diets and also

produced in the body. Glutamate is utilized by enterocytes as an energy source followed by glucose (Wu et al., 1995). Alanine is produced from pyruvate using nitrogen from glutamate and is an important nitrogen carrier in blood circulation (Hasumi et al., 1996). Phenylalanine and tyrosine are essential amino acids and are present in the diet or produced from bacteria (Simmonds, 1950). Betaine can be either absorbed from dietary sources (McGregor et al., 2002; Schwahn et al., 2003) or be produced from choline in the body (Craig, 2004). Betaine has been found to be used by bacteria for their cellular osmotic homeostasis (REF)

In the present study when pigs consumed the slow digestible starch diet, portal acetate concentration was increased at the expense of glucose concentration. Lower glucose concentration in portal plasma of pigs fed slowly digestible starch diet is due to reduced starch digestion as is evident from the lower rate and extent of in vitro starch digestion. As energy production from SCFA including acetate is at least 14% lower than that from glucose (Livesey, 1992; Jorgensen et al., 1997) mainly due to lower ATP yield, the results shows that starches with low in vitro digestibility and high amylose starch is energetically less efficient than low amylose starch.

When pigs consumed rapidly digestible starch, the portal concentration of alanine was greater than when they consumed slow digestible starch. This indicates that glucose after absorption may have been converted into metabolites such as non essential amino acids in the enterocytes or PDV, a finding that is supported by our

previous findings that up to 30 - 35% of starch digested in the small intestine may not be recovered in portal vein as glucose, lactate and SCFA (Regmi et al., 2011). Although pigs consumed an equal amount of amino acids, when pigs consumed slowly digestible starch the portal concentration of essential and semi-essential amino acids such as threonine, valine and tyrosine was lower compared to when they consumed rapidly digestible starch. This indicates that low availability of glucose in the intestine may increase the uptake of essential amino acids by enterocytes or PDV. Hence adequate intestinal supply of glucose is necessary to maintain the adequate level of essential amino acids in the portal circulation.

In the present study, plasma betaine was related to the slowly digestible starch diet. A previous NMR-based metabolomics study with pigs also showed that a whole grain rye-based diet resulted in an increase in betaine content in the plasma (Bertram et al., 2006). Betaine has been linked to reducing risk factors of cardiovascular disease in humans (Konstantinova et al., 2008) and improved growth and feed efficiency in animal (Fernandez-Figares et al., 2002; Siljander-Rasi et al., 2003). Betaine reduces plasma level of homocysteine (Holm et al., 2004), a risk factor for cardiovascular disease (Craig, 2004) by converting homocysteine to methionine (Delgado-Reyes and Garrow, 2005). In addition, plasma betaine is inversely associated with serum non-HDL cholesterol, triglycerides, BMI, percentage of body fat, waist circumference, and blood pressure (Konstantinova et al., 2008). The above information indicates that starch with high amylose and low in vitro digestibility can increase plasma betaine thus

potentially improving human health. In the present study, increase in betaine in the plasma was not foreseen, so we did not measure dietary betaine content. Detailed understanding of betaine content in different dietary components including starch and pathways related to betaine metabolism is needed to understand how the slowly digestible starch diet increased plasma betaine concentration in pigs.

In conclusion, this study updated our understanding on plasma biomarkers that are influenced by dietary starch. The findings showed that NMR technique can be used to understand the qualitative effects of dietary treatments in the kinetics of metabolites' profile in portal and arterial plasma in swine. Starch chemistry affects the profile of NMR-measured metabolites in portal and arterial plasma samples. Starch with high amylose and low in vitro digestibility was energetically less efficient and increased plasma betaine concentration potentially beneficial for human health.

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Chapter 5. Starch with high amylose and low in vitro digestibility increases intestinal nutrient flow and microbial fermentation and selectively promotes bifidobacteria in pigs[3]

5.1 Introduction

Starch typically constitutes a major portion of diets for human and monogastric animals. Starch is primarily digested by pancreatic α-amylase and brush border disaccharide hydrolases in the small intestine. Starch with lower digestibility increases the digesta mass in the distal gut (Topping et al., 1997). Greater mass of digesta provides a favorable environment for microbial growth in the distal gut by providing nutrients to gut bacteria and protecting the bacteria against the products of digestion such as bile acids and free fatty acids that have bactericidal actions (Topping et al., 1997). Hence greater digesta mass may increase the diversity and population of gut bacteria and the production of SCFA including butyrate. Commensal gut bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp. establish an efficient barrier to the invasion and colonization of the gut by pathogenic bacteria (Gibson and Roberfroid, 1995). Butyrate improves immune surveillance (Roediger, 1990; Cummings and Englyst, 1991; Morita et al., 2004) and increases growth and differentiation of enterocytes (Roediger, 1990; Cummings and Englyst, 1991; Scheppach, 1994). These properties are key to a healthy gut, because they reduce risks of diseases such as colon cancer and inflammatory bowel disease (Scheppach et al., 2001; Bauer-Marinovic et al., 2006).

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Previously, diets containing starch with higher content of amylose and resistant starch (RS) increased digesta mass, SCFA production, and population of *Bifidobacterium* spp. and *Lactobacillus* group in the gut (Brown et al., 1997; Kleessen et al., 1997; Wang et al., 2002; Morita et al., 2004; Bird et al., 2007). Although effects of amylose and resistant starch on nutrient digestibility, and gut microbial fermentation have been reported (Higgins, 2004; Englyst and Englyst, 2005; Singh et al., 2010), the relationship among starch characteristics, nutrient flow and microbial activity and profile is not clearly understood.

Therefore, diets containing 4 purified starches with a wide difference in starch chemistry were fed to ileal-cannulated pigs to test the hypothesis that starch with high amylose and slow digestibility improves gut bacterial diversity, selectively increases the population of *Bifidobacterium* spp. and *Lactobacillus* group, and increases the enzymes involved in butyrate production pathways in the gut. The objectives of the present study were to evaluate: a) the role of starch chemistry on intestinal nutrient flow, ileal and fecal fermentation characteristics and bacterial profile, and b) interrelation among starch characteristics, fermentation characteristics and microbial population in pigs.

5.2 Methodology

5.2.1 Starch sources

Remyline AX-DR 0% amylose rice starch, Remy B7 20% amylose rice starch (Remy Industries, Leuven-Wijgmaal, Belgium), Nastar 35.5% amylose pea starch

(Cosucra Group Warconing, Warconing, Belgium), and Gelose 80% amylose corn starch (Penford Food Ingredients, Centennial, CO) samples were used. The starches were previously characterized for purity, amylose content, crystallinity, granule size (Regmi et al., 2011) and in vitro digestibility (van Kempen et al., 2010). The starches were considered rapidly digestible (S1), moderately rapidly digestible (S2), moderately slowly digestible (S3), and slowly digestible (S4) based on the rate of maximal in vitro starch digestion (S1, 1.06; S2, 0.73; S3, 0.38 and S4, 0.22%/min; van Kempen et al., 2010).

5.2.2 Animal and diets

The animal use protocol was approved by the Animal Care Committee of the University of Alberta, in accordance with the guidelines of the Canadian Council on Animal Care (Canadian Council on Animal Care, 1993). Eight crossbred barrows (Duroc sire x Large White /Landrace F_1;Genex Hybrid; Hypor) initial body weight (BW), 36.2 ± 1.9 kg; initial age, 91 ± 7 d) were surgically fitted with a simple T–cannula at the distal ileum, approximately 5 cm prior to the ileo-cecal sphincter (Sauer et al., 1983; de Lange et al., 1989). Pigs were housed in individual metabolism pens (1.2 × 1.2 m) allowing freedom of movement, visual contact to other pigs, and free access to drinking water. After a 10-day recovery, pigs consumed one of the 4 diets containing 70% purified starches (**Table 5.1**) in a double 4 × 4 Latin square design at 0800 and 1600 h. Titanium dioxide (TiO_2) was included in the diet as an indigestible marker. Daily feed allowance was adjusted to 3 times the maintenance energy requirement (3 × 461 kJ digestible

energy/kg $BW^{0.75}$; NRC, 1998). Each experimental period comprised 17 d including an adaptation of 10 d to the diets to avoid carry-over effects followed subsequently by a 3-d total collection of feces and a 4-d collection of ileal digesta. Freshly voided feces were collected using plastic bags attached to the skin around the anus. Freshly voided ileal digesta were collected using plastic tubing attached to the barrel of the cannula. Immediately after collection, samples for microbial analyses were stored at -80°C and for SCFA analyses at -20°C. Ileal digesta and feces were each pooled for each pig observation and sub-sampled before analyses.

Table 5.1 Ingredient composition of 4 starch diets

Ingredient	*g/kg of diet*
Starch[1]	700
Casein[2]	140
Fish meal[3]	74
Cellulose[4]	40
Canola oil	10
Limestone	10
$CaHPO_4$	8
NaCl	3
Mineral premix[5]	5
Vitamin premix[6]	5
TiO_2	3
K_2CO_3	2

[1]Four sources, either: a) Remyline AX-DR rice (Remy Industries, Leuven-Wijgmaal, Belgium); and b) Remy B7 rice (Remy Industries); c) Nastar pea (Cosucra Groupe Warcoing, Warcoing, Belgium); d) Gelose 80 corn (Penford Food Ingredients, Centennial, CO) were used to prepare diets containing S1, S2, S3, and S4 starches, respectively. [2]Calcium caseinate, American Casein Company, Burlington, NJ; [3]Menhaden fish meal, Omega Protein, Hammond, LA; [4]Solka-floc, International Fiber Corp., North Tonawanda, NY; [5]Provided per kg diet: Zn, 100 mg as $ZnSO_4$; Fe, 80 mg as $FeSO_4$; Cu, 50 mg as $CuSO_4$; Mn, 25 mg as $MnSO_4$; I, 0.5 mg as $Ca(IO_3)_2$; Se, 0.1 mg as Na_2SeO_3; [6]Provided per kg diet: retinol, 2.5 mg; cholecalciferol, 20.6 µg; dl-α-tocopherol, 2.7 µg; niacin, 35 mg; D-pantothenic acid, 15 mg; riboflavin, 5 mg; menadione, 4 mg; folic acid, 2 mg; thiamine,1 mg; D-biotin, 0.2 mg; vitamin B-12, 0.025 mg.

5.2.3 Chemical analyses

Diets, ileal digesta and feces were analyzed for dry matter (DM) by drying at 100°C in an oven for 5 h (method 934.01; AOAC, 2006). Diets and freeze-dried digesta and feces were finely ground in a centrifugal mill through a 1.0-mm mesh screen (Retsch, Haan, Germany) prior to analyses of starch (Megazyme kit; Megazyme International, Bray, Ireland), crude protein (CP) (FP-428 N determinator; Leco Corp., Saint Joseph, MI), and TiO_2 (spectrophotometry; AOAC method 975.21; AOAC, 2006). Fecal and ileal digesta SCFA (acetate, propionate, butyrate, isobutyrate, isovalerate, isopropionate, isocaproate, and caproate) were analyzed by gas chromatography (Htoo et al., 2007).

5.2.4 DNA extraction, quantitative PCR (*qPCR*) *and Denaturing Gradient Gel Electrophoresis* (*DGGE*)

Genomic DNA was extracted from ileal digesta and feces of pigs and qPCR analysis was performed according to methods described previously (Metzler-Zebeli et al., 2010). For DNA extraction, 200 mg of sample was suspended in 1 mL of TN150 buffer (10 mM Tris-HCl, 150 mM NaCl [pH 8.0]) in a sterile tube containing 300-400 mg of sterile zirconium beads (diameter, 0.1 mm). The suspension was vortexed and centrifuged at 14,600 × g for 5 min. The pellet was washed twice with 1 mL of TN150 buffer and was resuspended in 1 mL of TN150 buffer. The cells were lysed by physical disruption in a mini-bead beater (Biospec Products). After centrifugation at 14,600 × g for 5 min, 900 μL of the supernatant was extracted twice with 1 mL Tris EDTA (10 mM Tris, 1 mM EDTA [pH 8.5])-

saturated phenol, followed by extraction with an equal volume of chloroform-isoamyl alcohol (24:1). The nucleic acids were precipitated with 2 volumes of ethanol (-20°C) and 0.1 volume of 5 M potassium acetate and stored overnight at -20°C. The DNA was collected by centrifugation at 14,600 x g for 20 min at 4°C, dried at room temperature for 1 h and dissolved in 50 µL Tris EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.5]).

For qPCR, reaction mixtures consisting of 12.5 µl SYBR Green Mastermix (Applied Biosystem), 1 µl of primers (10 µM for bacterial groups and 20 µM for butyrate enzyme genes) (**Table 5.2**), and 1 µl of template DNA of ileal or fecal samples were prepared and the reaction was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) using Sequence Detection Software (Version 2.01, Applied Biosystems). Each reaction was run in duplicate in a volume of 25 µl in optical reaction plates sealed with optical adhesive film (Applied Biosystems). Amplification involved one cycle at 95°C for 5 min for initial denaturation followed by 40 cycles of denaturation at 95°C for 15 s, primer annealing at the optimal temperatures (**Table 5.2**) for 30 s and extension at 72°C for 30 s, one cycle of 95°C for 1 min, one cycle of 55°C for 1 min, and a stepwise increase of the temperature from 55 to 95°C (at 10 s per 0.5°C) to obtain melt-curve data. Data were collected at the extension step and melting curves were checked after amplification to make sure amplification results were correct. Standard curves were generated using serial dilutions of the purified and quantified PCR products generated by standard PCR using specific primers

(**Table 5.2**) and genomic DNA from pig digesta (Metzler-Zebeli et al., 2010). The detection limit was 10^2, 10^3, and 10^4 copy numbers/g wet digesta for the bacterial group-specific primers, butyryl-Coenzyme A (CoA) CoA transferase, and butyrate kinase primers, respectively.

The bacterial diversity was determined by PCR-DGGE that was carried out with the extracted DNA from feces (Ketabi et al., 2011). The hypervariable V3 regions (corresponding to nucleotides 339–539 in *Escherichia coli*) of the 16S RNA genes were amplified by using PCR with universal primers HDA1-GC and HDA-2 (Muyzer and Smalla, 1998; **Table 5.2**). The PCR program used was 1 cycle at 95°C for 5 min for initial denaturation, followed by 35 cycles of denaturation at 95°C for 30 s, at 58°C for primer annealing for 2 min, and extension at 72°C for 1 min. The amplified product was used for DGGE which was carried out in duplicate as described previously (Walter et al., 2000). Briefly, the DGGE was performed with the Dcode Universal Mutation Detection System (Bio-Rad) using a 6% polyacrylamide gel with a 30 to 55% gradient of 7.0 M urea and 40% (vol/vol) formamide that increased in the direction of electrophoresis. Electrophoresis was carried out in 1 × TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) buffer at 130 V and 60°C for about 4.5 h. The gel was stained with SYBR green nucleic acid gel stain (10^5 × dilution of the stock solution in 1 × TAE buffer; Invitrogen) for 1 h and viewed by UV transillumination. DGGE profiles were compared using similarity analysis with the Bionumeric software (Applied Maths, Sint-Martens-Latem, Belgium) was used for similarity analysis of the DGGE gels.

Table 5.2 Oligonucleotide primers used to profile ileal digesta and feces from pigs consuming 4 starch diets differing in rate of digestion

Targeted bacterial group	Olionucleotide sequence (5′ - 3′)	A_T[1]	Reference
Total bacteria (200 bp)	F: CGGYCCAGACTCCTACGGG R: TTACCGCGGCTGCTGGCAC	60	(Lee et al., 1996)
Lactobacillus group[2] (341 bp)	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	62	(Walter et al., 2001) (Heilig et al., 2002)
Enterococcus spp. (144 bp)	F: CCCTTATTGTTAGTTGCCATCATT R: ACTCGTTGTACTTCCCATTGT	60	(Rinttila et al., 2004)
Streptpcoccus spp. (485 bp)	F: AGAGTTTGATCCTGGCTCAG R: GTTAGCCGTCCCTTTCTGG	60	(Nubel et al., 1996) (Franks et al., 1998)
Bifidobacterium spp. (243 bp)	F: TCGCGTC(C/T)GGTGTGAAAG R: CCACATCCAGC(A/G)TCCAC	60	(Rinttila et al., 2004)
Enterobacteriaceae family (195 bp)	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	63	(Bartosch et al., 2004)
Bacteroides group[3] (140 bp)	F: GGTGTCGGCTTAAGTGCCAT R: CGGA(C/T)GTAAGGGCCGTGC	60	(Rinttila et al., 2004)
Clostridium cluster I (120 bp)	F: ATGCAAGTCGAGCGAKG R: TATGCGGTATTAATCTYCCTTT	60	(Rinttila et al., 2004)
Clostridium cluster IV (130 bp)	F: GCACAAGCAGTGGAGT R: CTTCCTCCGTTTTGTCAA	60	(Matsuki et al., 2004)
Clostridium cluster XIVa (438-441 bp)	F: AAATGACGGTACCTGACTAA R: CTTTGAGTTTCATTCTTGCGAA	60	(Matsuki et al., 2002)
HDA (318- 518 bp)	F: CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GGC ACG GGG GGA CTC CTA CGG GAG GCA GCA G R: GTATTACCGCGGCTGCTGGCA	58	(Muyzer and Smalla, 1998)
Butyrate kinase (301 bp)	F: GTATAGATTACTIRYIATHAAYCCNGG R: CAAGCTCRTCIACIACIACNGGRTCNAC	53	(Louis et al., 2004)
Butaryl-CoA CoA-transferase (530 bp)	F: GCIGAICATTTCACITGGAAYWSITGGCAYATG R: CCTGCCTTTGCAATRTCIACRAANGC	53	(Louis and Flint, 2007)

[1]Annealing temperature (in °C); [2]*Lactobacillus* spp., *Pediococcus* spp., *Weissella* spp., and *Leuconostosc* spp.;
[2]*Bacteroides-Prevotella-Porphyrmonas*; F = forward primer; R= reverse primer.

5.2.5 Calculations and statistical analysis

The following variables were calculated after the analysis of diet, ileal digesta and feces. Ileal digesta flow of nutrients (mg/g DM fed) = ((100 – ileal digestibility % of nutrient) x total daily intake of nutrient in g × 10)/ total daily DM intake in g. Feces output of nutrient (mg/g DM fed) = ((100 – total tract digestibility % of nutrient) x total daily intake of nutrient in g × 10)/total daily DM intake in g. Post-ileal nutrient digestion (mg/g DM fed) = (ileal digesta flow – feces output) of nutrient in mg/g DM fed. Post-ileal net CP yield (mg/g DM fed) = (feces output – ileal digesta flow) of CP in mg/g DM fed. Ileal digesta or feces total SCFA content (μmol/g DM fed) = (SCFA concentration in μmol in ileal digesta or feces × total amount of ileal digesta or feces in g) / total daily DM intake in g.

Data were analyzed using SAS (version 9.1; SAS Institute). The model included diet as a fixed effect and period and interaction of diet and period as random effects. Means were separated for diet using the PDIFF statement in the mixed model. Pigs were considered the experimental units and significance of difference was set at $P < 0.05$. All variables were tested by the Kolmogoroff-Smirnoff test for normal distribution. Values reported are means ± pooled SEM; SEM of individual means were reported in cases of unequal variance among means.

Principal component (PC) and linear discriminant analyses were performed using JMP (version 8.0.2; SAS Institute). For PC analysis, the loading plot of PC 1 and PC 2, the first 2 eigenvalues, were used to determine the correlation among post-

ileal variables and starch characteristics. The angles between the lines were used to describe the interrelationship. Simple linear and non linear regression analyses were performed using JMP to determine relationships among variables. Ileal starch flow was log transformed when regressed against maximum rate of in vitro starch digestion to create a better fit.

5.3 Results

5.3.1 Food intake and body weight gain

All 4 starch diets were consumed promptly by pigs. Although feed intake did not differ among the pigs fed different starch diets, weight gain and feed efficiency (gain/feed) were greater when pigs consumed S2 and S3 diets than when pigs consumed S4 diet ($P < 0.05$) (**Table 5.3**).

5.3.2 Intestinal nutrient flow

When pigs consumed the S4 diet, ileal digesta flow of DM and starch, post-ileal digestion of DM and starch, and fecal output of DM, starch, and CP were greater ($P < 0.01$) but ileal CP flow was lower ($P < 0.01$) than when pigs consumed the remaining 3 starch diets (**Table 5.3**). In addition, ileal starch flow and post-ileal starch digestion was greater ($P < 0.01$) when the pigs consumed S3 diet than that when they consumed S1 and S2 diets.

Table 5.3 Growth and intestinal nutrient flow of pigs consuming 4 starch diets differing in rate of digestion[1]

Characteristic	Starch diets				Pooled SEM	*P* value
	S1	S2	S3	S4		
Growth						
Daily weight gain, g	718^{ab}	760^{a}	762^{a}	624^{b}	35.0	0.033
Feed efficiency (gain/feed), %	44.9^{ab}	49.8^{a}	48.3^{a}	38.7^{b}	2.55	0.025
Ileal digesta						
DM content, mg/g	122^{b}	125^{b}	132^{b}	244^{a}	9.1	<0.001
DM flow, mg/g DM fed[2]	24.5^{b}	25.6^{b}	35.6^{b}	291^{a}	4.26	<0.001
Starch flow, mg/g DM fed[3]	1.43^{c}	1.32^{c}	14.6^{b}	211^{a}	1.71	<0.001
CP flow, mg/g DM fed	13.0^{a}	12.4^{ab}	10.7^{b}	7.64^{c}	0.81	0.006
pH	7.53^{a}	7.50^{a}	7.04^{b}	6.62^{c}	0.097	<0.001
Feces						
DM content, mg/g	531^{a}	519^{a}	492^{ab}	433^{b}	19.3	0.002
DM output, mg/g DM fed	64.9^{b}	64.0^{b}	62.9^{b}	107.0^{a}	3.69	<0.001
Starch output, mg/g DM fed[4]	0.32^{b}	0.32^{b}	0.34^{b}	2.49^{a}	0.15	<0.001
CP output, mg/g DM fed	15.1^{b}	14.3^{b}	14.7^{b}	24.8^{a}	1.34	<0.001
Post-ileal DM digestion, mg/g DM fed[5]	-40.4^{b}	-38.3^{b}	-27.3^{b}	184^{a}	6.75	<0.001
Post-ileal starch digestion, mg/g DM fed[6]	1.11^{c}	1.03^{c}	14.3^{b}	209^{a}	1.64	<0.001
Post-ileal net CP yield mg/g DM fed[7]	2.10^{b}	1.90^{b}	3.98^{b}	17.1^{a}	1.11	<0.001

[1]Values are means and pooled SEM; n = 8; means in a row without a common superscript differ, $P < 0.05$. The SEM for the individual means for S1, S2, S3, and S4 diets was, respectively: [2]ileal digesta DM flow; 2.24, 1.82, 1.55, and 7.16; [3]ileal digesta starch flow; 0.35, 0.15, 1.50, and 6.14; [4]feces starch output; 0.03, 0.04, 0.02, and 0.32; [5]feces post-ileal DM digestion; 3.97, 2.46, 3.01, and 9.03; [6]feces post-ileal starch digestion; 0.36, 0.13, 1.51, and 6.26; and [5]feces post-ileal net CP yield; 0.56, 0.59, 0.76, and 1.42.

5.3.3 Fermentation characteristics

When pigs consumed the S3 diet, pH of ileal digesta was higher ($P < 0.01$) than when they consumed S4 diet but was lower ($P < 0.01$) than when they consumed S1 and S2 diets (**Table 5.3**). When pigs consumed the S1 diet, concentration (on wet basis) of acetate, propionate, butyrate, total SCFA and the branched-chain fatty acids (BCFA) isobutyrate and isovalerate in ileal digesta were higher ($P <$

0.01) than when they consumed the S4 diet (**Table 5.4**). The fecal concentration of acetate, propionate, butyrate, BCFA, and total SCFA did not differ when pigs consumed the 4 diets. However, the fecal concentration of valerate and caproate was higher ($P < 0.01$) when pigs consumed the S4 diet compared to that when they consumed the remaining 3 diets. Post-ileal net CP yield was higher ($P < 0.01$) when pigs consumed the S4 diet than when they consumed the remaining 3 diets (**Table 5.3**). The concentration per g of wet digesta was not different among diets, however, expression of fecal SCFA relative to DM intake showed that acetate, propionate, butyrate, and total SCFA content in feces (per g of DM fed) was higher ($P < 0.01$) when pigs consumed S4 diets than when they consumed the remaining 3 diets (**Figure 5.1**). Acetate and total SCFA content in ileal digesta (per g of DM fed) was higher ($P < 0.01$) when pigs consumed S4 diet compared to when they consumed the other 3 diets. Total propionate content in ileal digesta (per g of DM fed) was higher when pigs consumed the S1 diet than when they consumed S2 and S3 diets (**Figure 5.1**). The gene copy numbers of butyrate kinase and butyryl-CoA CoA transferase enzymes did not differ among pigs when they consumed different 4 starch diets (**Table 5.5**).

Table 5.4 SCFA concentration in ileal digesta and feces of pigs consuming 4 starch diets differing in rate of digestion[1]

Characteristic	Starch diets				Pooled SEM	*P* value
	S1	S2	S3	S4		
	µmol/g wet basis					
Ileal digesta						
Acetate	39.3[a]	21.0[b]	23.3[b]	18.8[b]	4.50	0.045
Propionate	6.12[a]	2.19[b]	2.12[b]	1.38[b]	0.776	0.006
Butyrate	2.93[a]	1.20[ab]	1.16[ab]	0.49[b]	0.409	0.017
Valerate	0.28	0.15	0.10	0.10	0.065	0.17
Caproate	0.01	0.01	0.03	0.04	0.009	0.12
Isovalerate	0.96[a]	0.41[b]	0.18[bc]	0.07[c]	0.089	<0.001
Isobutyrate	0.66[a]	0.25[b]	0.10[b]	0.10[b]	0.060	<0.001
Total	50.2[a]	25.2[ab]	27.0[ab]	21.0[b]	5.576	0.024
Feces						
Acetate	40.1	32.8	33.6	39.4	3.41	0.69
Propionate	8.01	6.55	8.05	9.50	0.792	0.06
Butyrate	6.22	5.04	7.98	6.74	1.277	0.40
Valerate	1.23[b]	0.87[b]	1.26[b]	3.69[a]	0.387	<0.001
Caproate	0.34[b]	0.34[b]	0.23[b]	2.10[a]	0.191	<0.001
Isovalerate	1.40	1.23	1.78	2.51	0.317	0.09
Isobutyrate	1.98	1.77	2.69	3.83	0.558	0.12
Total	59.3	48.6	56.9	67.8	5.39	0.28

[1]Values are means and pooled SEM; $n = 8$; means in a row without a common superscript differ, $P < 0.05$.

5.3.4 Microbial profile

Gene copy numbers (wet basis) of total bacteria, *Lactobacillus* group, *Enterococcus* spp., *Streptococcus* spp., *Enterobacteriaceae*, *Bacteroides* group, and *Clostridium* clusters I, IV and XIVa in ileal digesta and feces were not different when pigs consumed different 4 diets (**Table 5.5**). Gene copy numbers (wet basis) of *Bifidobacterium* spp. in feces, but not in ileal digesta, was higher ($P < 0.05$) when pigs consumed the S4 diet than when they consumed the remaining 3 diets (**Table 5.5**). Otherwise, the profile of bacteria as shown by DGGE using 16S rDNA was not different when pigs consumed the four different starch diets (**Figure 5.2**).

Table 5.5 Bacterial groups in ileal digesta and feces and butyrate-production pathway of pigs consuming 4 starch diets differing in rate of digestion[1]

Characteristic	Starch diets				Pooled SEM	*P* value
	S1	S2	S3	S4		
Bacterial groups in ileal digesta, log_{10} 16S rRNA gene copies/g wet wt						
Total Bacteria	9.62	9.69	10.02	9.97	0.175	0.31
Lactobacillus group	7.91	7.44	7.62	7.57	0.297	0.74
Enterococcus spp.	8.76	9.02	9.19	9.18	0.282	0.69
Streptococcus spp.	8.72	9.13	9.13	9.14	0.434	0.71
Bifidobacterium spp.	5.52	5.39	5.41	5.61	0.269	0.93
Enterobacteriaceae	8.63	8.81	9.30	9.08	0.319	0.48
Bacteroides group	7.91	7.85	8.01	7.40	0.183	0.13
Clostridium cluster I	7.73	7.52	7.81	7.54	0.201	0.45
Clostridium cluster IV	6.48	6.33	6.56	6.35	0.198	0.83
Clostridium cluster XIVa	7.50	7.32	7.42	6.85	0.218	0.19
Bacterial groups in feces, log_{10} 16S rRNA gene copies/g wet wt						
Total Bacteria	9.51	9.63	9.52	9.55	0.961	0.25
Lactobacillus group	5.63	5.65	5.57	6.28	0.209	0.26
Enterococcus spp.	7.83	8.30	7.56	6.90	0.433	0.06
Streptococcus spp.	6.46	6.97	6.91	6.63	0.256	0.48
Bifidobacterium spp.	5.57^{b}	5.23^{b}	5.11^{b}	6.58^{a}	0.372	0.047
Enterobacteriaceae	8.19	8.51	8.36	7.88	0.333	0.60
Bacteroides group	9.19	9.34	9.19	8.71	0.197	0.17
Clostridium cluster I	8.17	8.49	8.04	8.39	0.270	0.63
Clostridium cluster IV	8.19	8.54	8.38	8.16	0.248	0.69
Clostridium cluster XIVa	7.26	7.85	7.21	6.84	0.293	0.15
Enzymes, *log10 gene copies/g wet wt*						
Butyrate kinase	6.43	6.69	6.29	6.61	0.142	0.48
Butyryl-CoA CoA transferase	5.15	5.21	4.97	5.19	0.196	0.77

[1]Values are means and pooled SEM; $n = 8$; means in a row without a common superscript differ, $P < 0.05$.

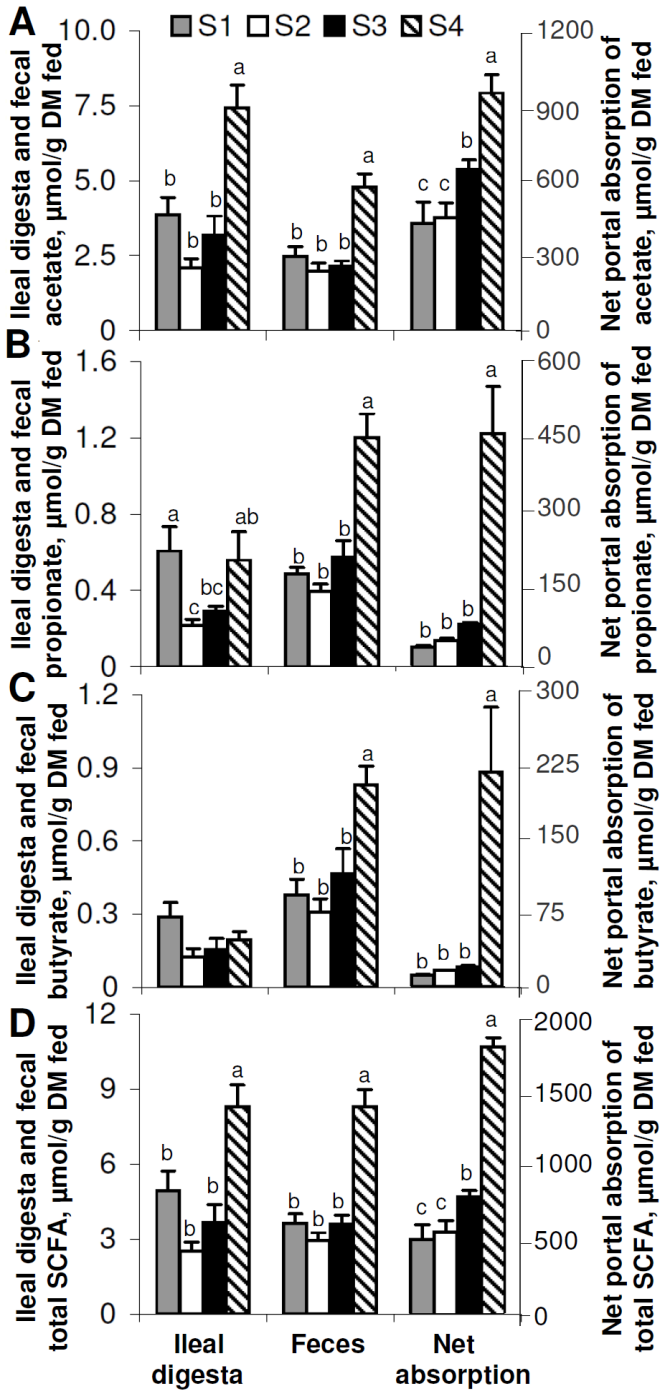

Figure 5.1 Ileal digesta and fecal total content and net absorption of acetate (**A**), propionate (**B**), butyrate (**C**), and total SCFA (**D**) in pigs consuming 4 starch diets differing in rate of digestion; $n = 8$. Net portal absorption of SCFA was calculated from a study with portal-vein catheterized pigs consuming the same 4 diets (Regmi et al., 2011) using the following 2 formulas: net portal absorption$n$ = (SCFA concentration in portal vein – SCFA concentration in carotid artery) × plasma flow rate in the portal vein and net portal absorption of SCFA/g DM fed = 2 × cumulative 12 h net portal absorption of SCFA/daily DM intake in g. Values are means and SEM; means without a common letter differ, $P < 0.05$.

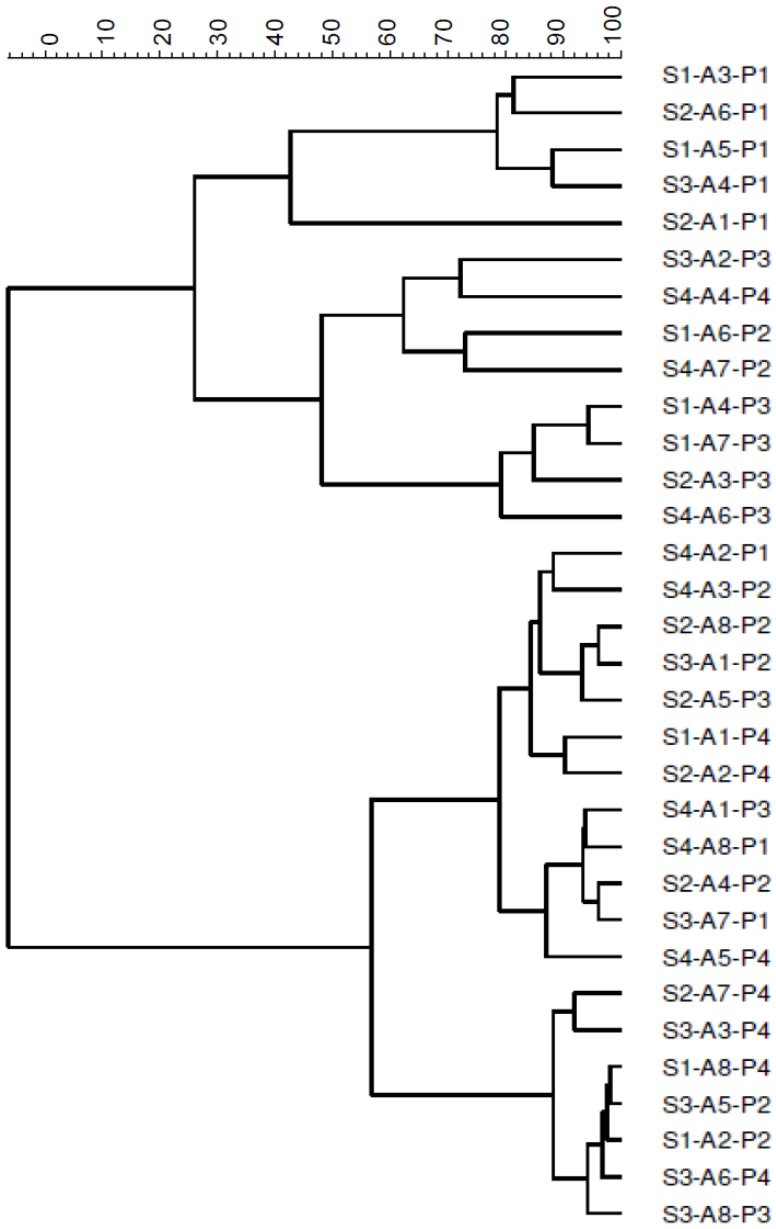

Figure 5.2 Bacterial diversity in feces of pigs consuming 4 starch diets differing in rate of digestion as determined by DGGE using HDA primers; S1-S4, Starch diets; A1-A8, pigs; P1-P4, periods; n = 8.

5.3.5 Interrelation among starch characteristics, nutrient flow, fermentation, and microbial profile

The rate of in vitro starch digestion (van Kempen et al., 2010) had a negative quadratic relationship (R^2 = 0.98; P < 0.001) with the daily total ileal starch flow and flow reached a plateau at 0.8%/min of maximal rate of in vitro digestion (**Figure 5.3**). Similarly, total daily ileal starch flow had a linear (P < 0.01) relationship with total population of *Bifidobacterium* spp. and *Lactobacillus* group, total daily fecal butyrate content, and total daily post-ileal net CP yield but was not related (P = 0.92) to the total daily population of *Enterobacteriaceae* (**Figure 5.4**).

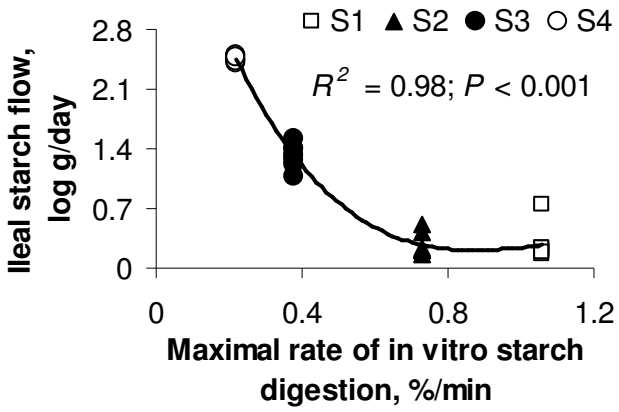

Figure 5.3 Relation of in vitro starch digestion rate with ileal starch flow in pigs consuming 4 starch diets differing in rate of digestion; n = 8.

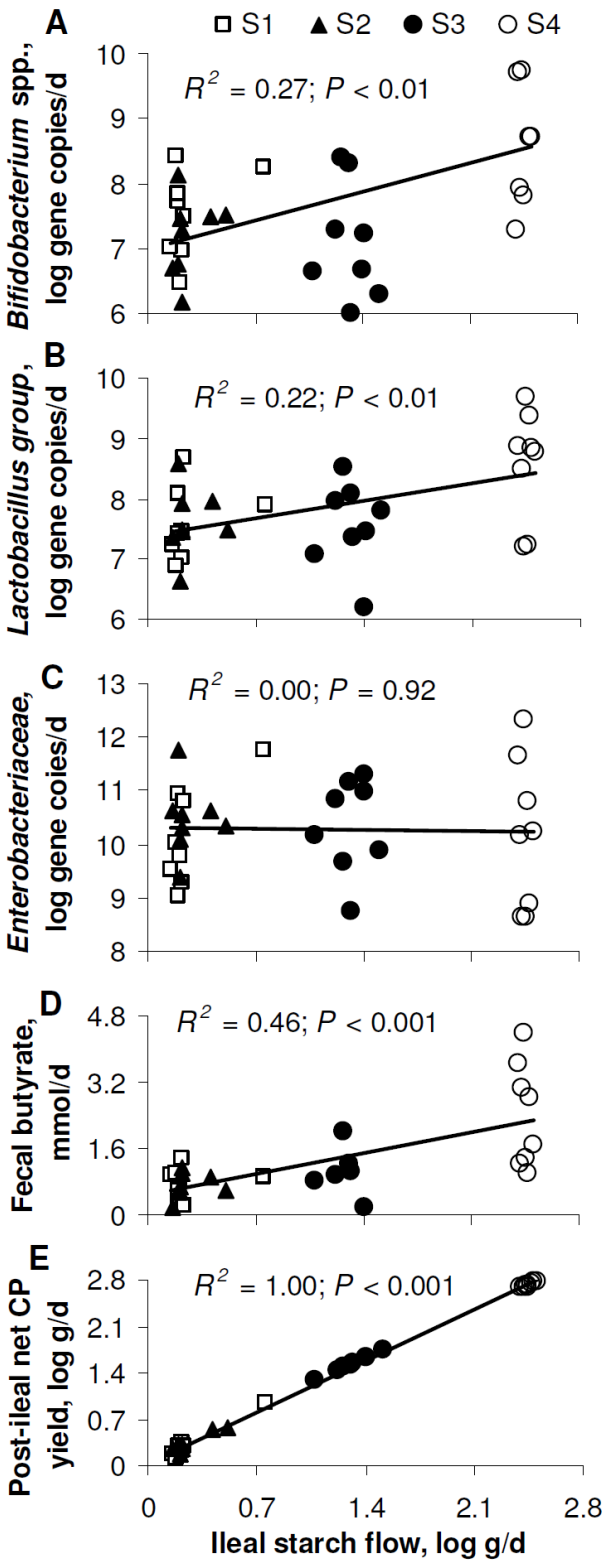

Figure 5.4 Relation of ileal starch flow with population of *Bifidobacterium* spp. (**A**), *Lactobacillus* group (**B**) and *Enterobacteriaceae* (**C**); fecal butyrate (**D**); and post-ileal net CP yield (**E**) in pigs consuming 4 starch diets differing in rate of digestion; n = 8.

Linear discriminant analysis showed that the S4 diet clustered distinctly different compared to remaining 3 diets. The S4 diet was more related to total bacteria, *Lactobacillus* group, and *Bifidobacterium* spp. in feces; whereas, the remaining 3 diets were more related to *Enterococcus* spp., *Enterobacteriaceae* and *Costridium* cluster IV in feces (**Figure 5.5**). Loading plot from PCA showed that amylose content had strong positive relation (small angle among variables) and in vitro starch digestion rate and plateau had strong negative relation (angle close to 180^0 among variables) with ileal starch flow, post-ileal starch digestion, butyrate, propionate, total SCFA, *Lactobacillus* group, *Bifidobacterium* spp. (**Figure 5.6**). Similarly, ileal starch flow was positively related with the gene copy numbers of butyrate production pathway enzymes, total bacteria, *Lactobacillus* group, and *Bifidobacterium* spp. Post ileal net CP yield was positively related to total BCFA.

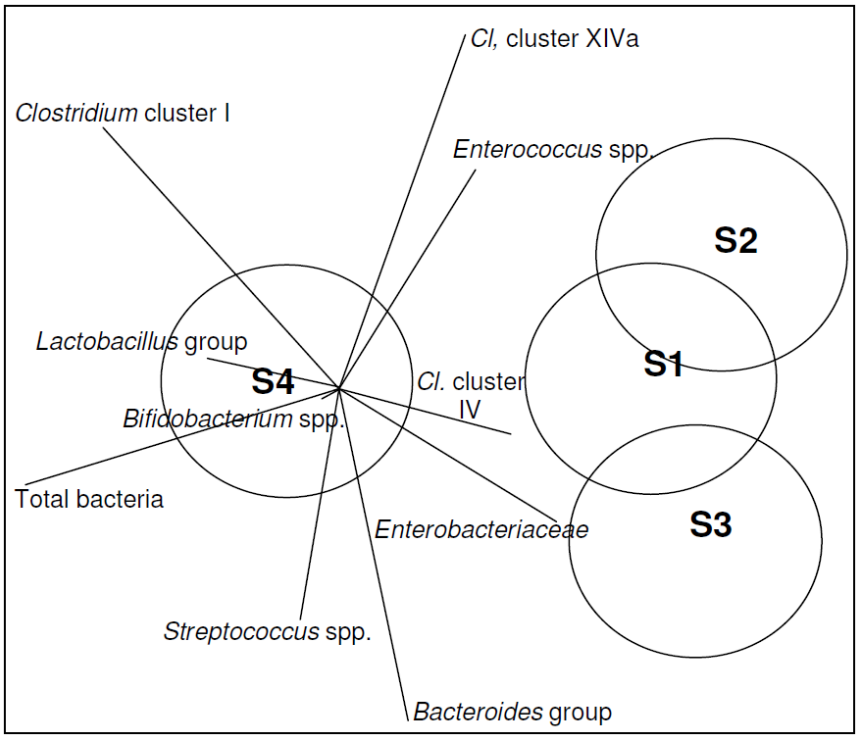

Figure 5.5 Linear discriminant analysis of gene copies of bacterial groups in feces of pigs consuming 4 starch diets differing in rate of digestion; S1-S4, starch types.

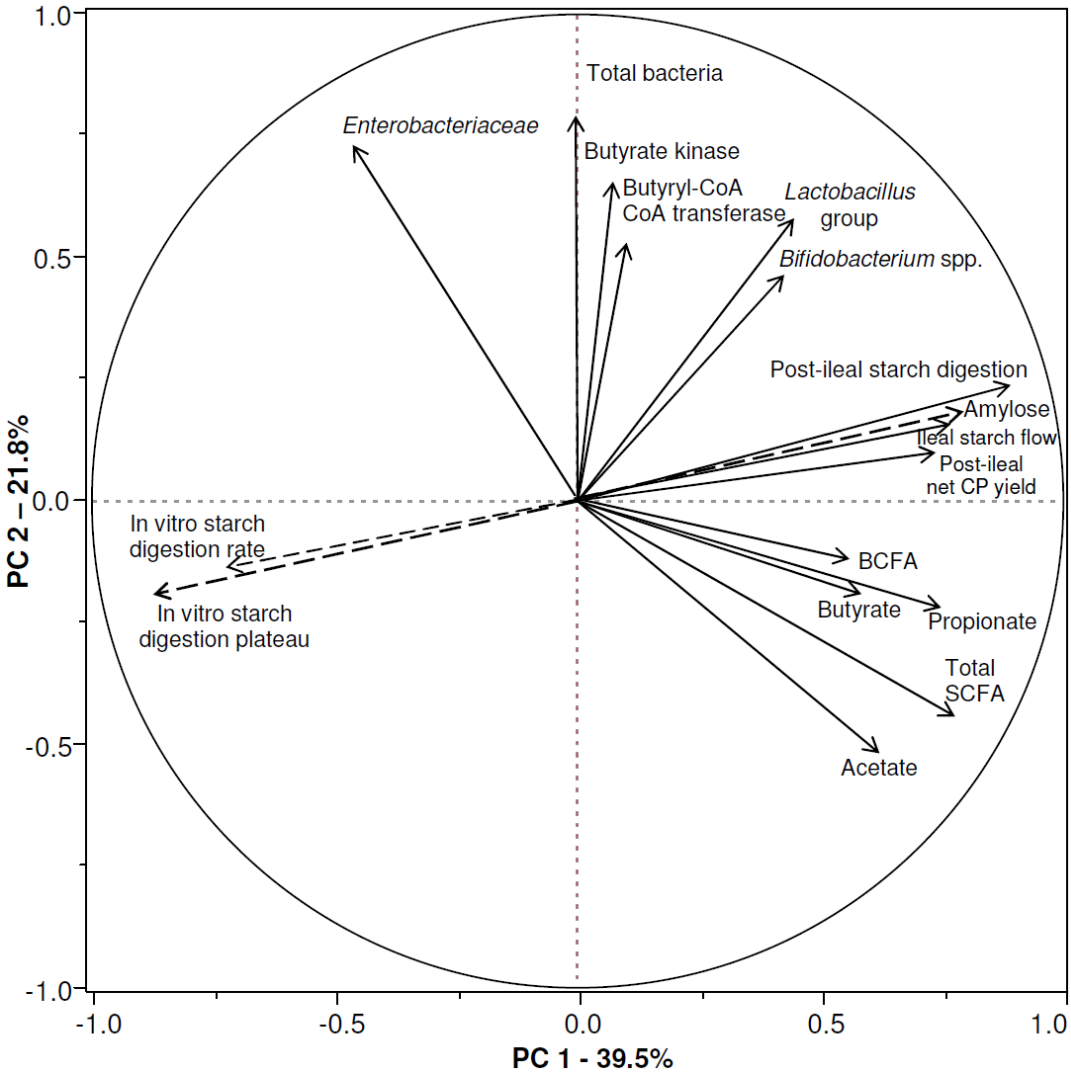

Figure 5.6 Loading plot showing the correlations among starch characteristics (dashed arrows), gene copies of major bacterial groups in feces and butyrate production pathway enzymes in feces, fecal SCFA, and intestinal nutrient flow and digestibility (solid arrows) of the first two eigenvalues (PC 1 and PC 2) in pigs consuming 4 starch diets differing in the rate of digestion; values except starch characteristics are based on per g DM fed, n = 8.

5.4 Discussion

The present study evaluated the effects of purified starches with a wide range in physicochemical properties on post-ileal nutrient flow, fermentation characteristics, and microbial profile in the gut. Previously, diets containing high amylose starch and resistant starch (RS) increased post-ileal digesta flow and microbial fermentation in the distal gut (Pluske et al., 2007). However, whether these effects were solely due to starch or confounded by starch-associated compounds such as protein, fat, and fiber present in grains or diets was not clear. In addition, amylose or RS alone may not be an accurate indicator of in vivo biological responses including microbial fermentation and population and digesta mass (Bird et al., 2007; van Kempen et al., 2010).

In the present study, high levels of (70%) purified starches instead of intrinsic starch were used in diets to minimize confounding effects of starch-associated compounds. Moreover, the starches were characterized in depth and in vitro starch digestion was used as a link between physicochemical properties of starch and in vivo responses (van Kempen et al., 2010). The ileal-cannulated pig model was used to measure nutrient availability for microbial fermentation in the large intestine and to differentiate nutrient digestibility in the large vs. small intestine. Pigs and humans have similar digestive physiology and anatomy (Pond and Lei, 2001) except for the cecum in pigs as opposed to the appendix in humans. Moreover, pigs and humans have a similar profile of carbohydrate-derived nutrients and relatively similar profile of gut microbiota (Lee et al., 2010); thus,

pigs can be used to understand the intestinal nutrient flow and microbial fermentation and profile in humans (Spurlock and Gabler, 2008).

5.4.1 Animal performance and intestinal nutrient flow

Both growth rate and feed efficiency was lower when pigs consumed the S4 diet compared to when pigs consumed the S2 and S3 diets. Previously, feed efficiency was either decreased (de Deckere et al., 1995; Kim et al., 2003) or unaffected (de Deckere et al., 1993; Verbeek et al., 1995; Kishida et al., 2001; Bird et al., 2007) by starches with high amylose and RS content. The reduced enzymatic digestion and increased fermentation of starch might have contributed to lower energetic efficiency, thus resulting in lower feed efficiency when pigs consumed starches with slow rates of in vitro digestion in the present study. Finally, removal of digesta for 4 out of 17 days per experimental period had a greater impact when pigs consumed S4 diet because more starch and energy was contained in this digesta.

Nutrient flow into the distal gut and intestinal digesta mass are considered to be important factors influencing digesta passage rate and microbial activity in the gut (Phillips et al., 1995). Starch diets with low in vitro digestibility increased nutrient flow into the large intestine and also doubled fecal bulk (Saito et al., 2001; Bird et al., 2007; Bird et al., 2009, present study). Bulk-forming property of slow digested starch might be too low compared to cellulose (Takeda and Kiriyama, 1979) to be effective to reduce the risk of constipation. Previously, diets

containing starch with high amylose and RS decreased ileal CP flow (Sun et al., 2006), but increased fecal CP output (Kishida et al., 2001; Sun et al., 2006) indicating increased small intestinal CP digestion similar to the findings in the present study.

5.4.2 Microbial fermentation and butyrate enzymes

Previously, diets with higher content of RS and high amylose starch increased SCFA concentration in the distal gut (Jenkins et al., 1998; Bird et al., 2007; Bird et al., 2009). When pigs consumed the S4 diet, the concentration of individual and total SCFA did not increase (this study); however, the total content, i.e., production (this study) and net portal absorption (**Figure 5.1,** Regmi et al., 2011) of total and individual SCFA on a daily basis increased notably compared to that when they consumed the remaining 3 diets. As the concentration of SCFA in the gut is affected by both microbial fermentation and the rate of absorption of SCFA (Cummings and Macfarlane, 1991); increased production of SCFA might not be reflected solely by increased SCFA concentration in the gut.

Although numerous species belonging to the *Clostridium* clusters can utilize starch to produce butyrate (Barcenilla et al., 2000; Sharp and Macfarlane, 2000), specific butyric acid producing phylogenic clusters of bacteria have not yet been identified (Louis et al., 2007). Hence, the population of butyrate-producing bacteria in the gut is difficult to estimate using currently available techniques. Estimations of key enzymes of butyrate production pathways such as butyrate

kinase and butyryl-CoA CoA transferase (Diez-Gonzalez et al., 1999; Louis et al., 2004) can provide useful information on the activity of butyrate-producing bacteria in the gut. In the present study the gene copy numbers of both butyrate kinase and butyryl-CoA CoA transferase per g of fecal samples and *Clostridium* clusters on wet basis were not affected by the 4 starch diets indicating that starch chemistry may not play a major role in the butyrate production pathways in the distal gut.

In the present study, butyrate kinase and butyryl-CoA CoA transferase concentration expressed per g of feces did not differ among diets; however, total content of these enzymes in feces was greater when the pigs consumed slowly digestible starch compared to when the pigs consumed the other 3 starch diets (data not shown). These data parallel the butyrate data indicating that butyrate per g of feces did not differ among diets, but total butyrate content in feces was greater when the pigs consumed slowly digestible starch compared to when the pigs consumed the other 3 starch diets.

Greater post-ileal net CP yield and population of total gut bacteria in feces (on a daily basis) indicated that microbial protein production is increased when pigs consumed the S4 diet compared to remaining 3 diets. In addition, a strong positive relation between total fecal BCFA and post-ileal net CP yield indicated that a portion of CP was fermented in the large intestine of pigs consuming the S4 diet.

5.4.3 Microbial profile and diversity

Nutrient flow into the distal gut serves as a substrate for gut microflora. In the present study, population of major bacterial groups in ileal digesta was high, likely due to availability of digestible starch in the distal small intestine. The bacterial population in ileal digesta was dominated by facultative anaerobes (*Lactobacillus* group and *Enterobacteriaceae*) with low numbers of strict anaerobes, while the reverse was observed in fecal microbiota. In the present study, the S4 diet selectively promoted *Bifidobacterium* spp. in the large intestine without affecting the population of any other bacterial group. This finding was reflected by PCR-DDGE patterns indicating that starch diets did not affect microbial diversity. As *Bifidobacterium* spp. was present at < 0.04% of total bacterial population, their population change likely was not detected by PCR-DDGE. Previously, a starch diet with high amylose (80 vs. 0%) and RS (10 vs. 0% of starch content promoted the growth of *Bifidobacterium* spp. and *Lactobacillus* group in the distal gut of rats and pigs (Brown et al., 1997; Kleessen et al., 1997; Wang et al., 2002; Morita et al., 2004; Bird et al., 2007). In addition to high amylose starch, waxy starch can also stimulate the growth of *Bifidobacterium* spp. in the large intestine as long as starch flows into the large intestine (Wang et al., 2002). Several strains of *Bifidobacterium* possess α-amylase and pullulanase activity required for specific use of starch in the distal gut (Ryan et al., 2006). Availability of starch in the distal gut selectively increases the population of *Bifidobacterium* spp. and *Lactobacillus* group without increasing the population of total anaerobes and *Enterobacteriaceae* in feces and

digesta (Brown et al., 1997; Morita et al., 2004; Bird et al., 2007, this study). Hence, this study supports the previous concept that starch with low in vitro digestibility selectively promotes the population of beneficial gut microbes including *Bifidobacterium* spp. in monogastric animals and humans (Gibson and Roberfroid, 1995; Wang et al., 2002; Bird et al., 2007; Martinez et al., 2010).

5.4.4 Interrelationship among variables

In the present study, high amylose content and low in vitro starch digestibility in non-gelatinized starches are strongly related to the starch flow into the large intestine. From 0.22 to approximately 0.8%/min of maximal in vitro starch digestion rate, starch flow into the large intestine decreased and the flow reached a plateau beyond 0.8%/min maximal in vitro starch digestion rate. The positive relation of ileal starch flow with *Bifidobacterium* spp. and *Lactobacillus* group and no relation with *Enterobacteriaceae* as shown by regression and linear discriminant analysis clearly indicates that starch available at the distal gut can indeed selectively promote the population of potentially beneficial gut bacteria without affecting (this study) or possibly reducing the population of specific phylogenic group such as *Enterobacteriaceae* which contains many potentially harmful bacterial species (de la Cal et al., 2005; Schierack et al., 2007). Ileal starch flow is strongly related to production of SCFA including butyrate, and key butyrate production pathway enzymes. In addition, these relationships indicate that greater than 35 g ileal starch flow per day, which is equivalent to 0.36%/min maximal rate of in vitro digestion or 40% amylose content, is required to have

beneficial effects of dietary starch on bifidobacteria and butyrate production in the gut. This value is much more than the current RS recommendation for humans (20 g/d; Baghurst et al., 1996).

In conclusion, starch with high amylose and low *in vitro* digestibility increased post-ileal nutrient flow and microbial fermentation, and selectively promoted *Bifidobacterium* spp. in the distal gut. Substantial changes of starch chemistry (at least ≥40% amylose content and ≤0.36%/min in vitro digestion rate) were required to have potentially beneficial changes in the post-ileal variables in pigs.

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Chapter 6. Starch with high amylose and low in vitro digestibility increases ileal flow and post-ileal flux of macro- and trace minerals without affecting retention in pigs

6.1 Introduction

Starch containing higher proportion of slowly digestible starch (SDS) and resistant starch (RS) fractions are considered beneficial for the prevention and management of diseases related to carbohydrate metabolism (Behall et al., 2006; Grabitske and Slavin, 2008) and are, thus, used in many food preparations (Grabitske and Slavin, 2008). In monogastric animals such as pigs, SDS and RS may beneficially influence indicators of fat metabolism that may influence lean vs. fat deposition, thus, meat quality (Higgins et al., 2004; de Deckere et al., 1993; de Deckere et al., 1995; Kishida et al., 2001; Kim et al., 2003). These starch fractions may affect the solubility and bacterial utilization of minerals and absorptive surface area of gut epithelium (Schulz et al., 1993; Younes et al., 1996; Hrdina et al., 2009) and thus, may modulate absorption and body retention of minerals (Abrams et al., 2007; Demigne et al., 2008). Thus, a need exists to define the role of starch chemistry on mineral absorption in the small intestine and to elucidate the consequences of starch fermentation on mineral absorption in the large intestine of monogastric species.

Mineral availability in the large intestine can improve gut health exerting a protective effect on the colon epithelium (Wargovich et al., 1983) and inhibit the cytotoxicity of potential carcinogens (Govers and Van der Meet, 1993). These

effects could be due to greater gut bacterial growth and fermentation likely resulted from utilization of minerals by the bacteria as nutrients (Hrdina et al., 2009; Metzler et al., 2009).

The hypothesis was that starch chemistry affects intestinal absorption and body retention of minerals. The objective of the present study was to understand the effects of starch chemistry on a) ileal flow and apparent retention of minerals and b) the relation between fecal SCFA, fecal numbers of bacterial groups and post-ileal mineral and nitrogen flux in pigs fed starch diets with different rates of in vitro digestion.

6.2 Materials and Methods

6.2.1 Animals and diets

The animal use protocol was approved by the Animal Care Committee of the University of Alberta, in accordance with the guidelines of the Canadian Council on Animal Care (1993). Eight crossbred barrows (Duroc sire x Large White /Landrace F_1;Genex Hybrid; Hypor, Regina, SK) initial BW, 36.2 ± 1.9 kg; initial age, 91 ± 7 d) were surgically fitted with a simple T–cannula at the distal ileum, approximately 5 cm prior to the ileo-cecal sphincter. The preparation of the cannulas, surgical procedure, and modifications were described previously (Sauer et al., 1983; de Lange et al., 1989). Pigs were housed in individual metabolism pens (1.2 × 1.2 m) allowing freedom of movement, visual contact to other pigs, and free access to drinking water.

After a 10-day recovery, pigs consumed one of the 4 diets containing 70% purified starches (**Table 5.1**) in a double 4 × 4 Latin square design at 0800 and 1600 h. The starches used in the study were Remyline AX-DR 0% amylose rice starch, Remy B7 20% amylose rice starch (Remy Industries, Leuven-Wijgmaal, Belgium), Nastar 35.5% amylose pea starch (Cosucra Group Warconing, Warconing, Belgium), and Gelose 80% amylose corn starch (Penford Food Ingredients, Centennial, CO). The starches were previously characterized for purity, amylose content, crystallinity, granule size, CP content, crude fat content (Regmi et al., 2011) and in vitro digestibility (van Kempen, et al., 2010). The starches were considered rapidly digestible (S1), moderately rapid digestible (S2), moderately slow digestible (S3), and slowly digestible (S4) based on the rate of maximal in vitro starch digestion (S1, 1.06; S2, 0.73; S3, 0.38 and S4, 0.22%/min; van Kempen, et al., 2010). The amounts of Ca, Mg, P, Cu, Fe, Mn, and Zn originating from the non-starch part of the diet were similar among diets. Titanium dioxide (TiO_2) was included in the diet as an indigestible marker. Daily feed allowance was adjusted to 3 times the maintenance energy requirement (3 × 110 kcal DE/kg $BW^{0.75}$; NRC, 1998). Each experimental period comprised 17 d including an adaptation of 10 d to the diets followed subsequently by a 3-d total collection of feces and urine and a 4-d collection of ileal digesta. Freshly voided feces were collected using plastic bags attached to the skin around the anus. Urine was collected in boxes containing 60 mL of concentrated sulphuric acid and stored at -20°C. Freshly voided ileal digesta were collected using plastic tubing

attached to the barrel of the cannula. Thereafter, ileal digesta and feces were pooled for each pig, sub-sampled, and stored at -80°C for quantification of bacterial groups and at -20°C for SCFA determination.

6.2.2 Analytical Methods

Diets and freeze-dried digesta and feces were finely ground in a centrifugal mill through a 1.0-mm mesh screen (Retsch, Haan, Germany) prior to analyses of starch (Megazyme kit; Megazyme International Ireland, Ltd., Bray, Ireland), N (FP-428 N determinator; Leco Corp., St. Joseph, MI), and TiO_2 (spectrophotometry; AOAC method 975.21; (AOAC, 2006). Phosphorus in feed, digesta, feces, and urine was analyzed spectrophotometrically at 400 nm using the vanadate-molybdate method (method 946.06; AOAC, 2006). The Ca, Mg, Cu, Fe, Mn, and Zn content in feed, digesta, feces, and urine was analyzed by dry ashing the samples (method 968.08, AOAC, 2006) followed by atomic absorption spectrometry (Varian SpectAA 240 FS, Mississauga, Ontario, Canada). The Ca and Mg content in feed, digesta, feces, and urine were analyzed after adequate dilution into 0.1% (w/v) lanthanum chloride. Phytate content in diets was analyzed at the University of Missouri, Columbia, MO (method 32.5.18; AOAC, 2006). The 16S rRNA gene copy number of bacterial groups (i.e., total bacteria, *Lactobacillus spp., Enterococcus spp., Streptococcus spp., Bifidobacterium spp., Clostridium* cluster I, IV, and XIVa, *Bacteroides-Prevotella-Porphyromonas* group, and *Enterobacteriaceae*) in feces was quantified after phenol-chloroform DNA extraction using a 7500 Fast Real-Time PCR System (Applied Biosystems,

Foster City, CA; for details see (Chapter 5). Fecal and ileal digesta SCFA (acetate, propionate, butyrate, isobutyrate, isovalerate, isopropionate, isocaproate, and caproate) were analyzed by gas chromatography (Htoo et al., 2007). After analysis, following parameters were calculated using respective formula:

Ileal mineral flow (*% of mineral intake*) =100 – ileal digestibility% [Eq. 1]

Fecal excretion, (*% of mineral intake*) = 100 – total tract digestibility% [Eq. 2]

Urinary excretion, (*% of mineral intake*) = urinary excretion*100/daily mineral intake [Eq. 3]

Retention (*g or mg/d*) = daily mineral intake – (fecal excretion + urinary excretion) in g or mg [Eq. 4]

Retention, (*% of mineral intake*) = (daily mineral intake – (fecal excretion + urinary excretion))*100/ daily mineral intake [Eq. 5]

6.2.3 Statistical analysis

Data were analyzed using SAS (version 9.1; SAS Inst. Inc., Cary, NC). The model included diet as a fixed effect and period and interaction of diet and period as random effects. Means were separated for diet using the PDIFF statement in the mixed model. Pigs were considered the experimental units and significance of difference was set at $P < 0.05$. All variables were tested by the Kolmogoroff-Smirnoff test for normal distribution. Values in the text are means ± SEM.

Principal component (PC) and linear discriminant analyses were performed using JMP (version 8.0.2; SAS Inst.). For PC analysis, the loading plot of PC 1 and PC 2, the first 2 eigenvalues, were used to determine the correlation among post-ileal variables and starch characteristics. The angles between the straight lines were used to describe the interrelationship. The length of individual straight line describes the variability in the data.

6.3 Results

Pigs recovered well from surgery and remained healthy throughout the study. The average BW of the pigs was 40.5, 49.3, 58.9, and 70.8 kg at the beginning of periods 1, 2, 3, and 4, respectively. The average BW of the pigs was 82.0 kg at the end of the study.

6.3.1 Feed DM and mineral intake

The average daily DM intake across the 4 experimental periods was 1.5±0.10, 1.5±0.09, 1.6±0.11, 1.6±0.10 kg/d, respectively, for pigs receiving S1, S2, S3, and S4 diets. Although the amounts of Ca, Mg, P, Na, K, Zn, Fe, Cu, and Mn originating from non-starch components of the diet were similar for all 4 diets, the starch fractions contributed differently to the mineral levels of the diets due to difference in intrinsic mineral profiles including phytate contents in the 4 purified starches (**Table 6.1**). As a result, daily intake of minerals except Na differed among pigs fed different starch diets (**Table 6.2, 6.3 and 6.4**). For instance, pigs fed S4 diet had lower ($P < 0.05$) intake of Ca, Mg, Fe, Cu and higher ($P < 0.05$) intake of K and Zn compared to pigs fed S1 diet.

Table 6.1 Analyzed mineral content of 4 purified starches and diets (DM basis)

Item	S1	S2	S3	S4
Starch				
DM, %	89.7	88.6	87.8	87.9
Ca, *mg/kg*	299.9	3.4	33.6	6.5
Mg, *mg/kg*	70.0	82.6	13.9	16.8
P, *mg/kg*	339.3	662.5	423.8	636.0
Phytate[1], *g/kg*	< 0.10	0.39	< 0.10	0.15
Na, *mg/kg*	401.0	759.4	257.8	102.9
K, *mg/kg*	18.4	164.3	19.1	46.5
Diet				
DM, %	90.2	89.5	90.1	89.5
Starch, g/kg	699.2	689.9	683.8	644.2
Ca, *g/kg*	10.38	8.69	8.15	8.48
Mg, *g/kg*	0.42	0.37	0.35	0.30
Total P, *g/kg*	5.41	5.16	4.28	5.01
Phytate, *g/kg*	2.61	6.27	2.91	4.44
Na, *g/kg*	2.50	2.49	2.35	2.10
K, *g/kg*	2.44	3.10	3.05	3.06
Zn, *mg/kg*	134.7	138.3	133.0	149.8
Fe, *mg/kg*	215.4	173.2	209.5	147.1
Cu, *mg/kg*	58.7	35.5	59.4	40.2
Mn, *mg/kg*	37.0	52.1	32.4	37.9

[1]Detection limit was 0.10 g/kg.

Table 6.2 Intake, ileal flow, postileal flux, fecal excretion, urinary excretion and apparent retention of Ca, Mg, P, Na, K, and N in pigs fed 4 starch diets[1]

Diet	S1	S2	S3	S4	Pooled SEM	*P* value
Ca						
Intake, *g/d*	15.3[a]	13.1[b]	12.6[b]	13.4[b]	1.15	0.003
Ileal flow, *% of intake*	0.72[b]	0.90[b]	1.02[b]	1.51[a]	0.120	0.014
Post-ileal flux, *% of intake*	-0.27[b]	-0.40[b]	-0.12[b]	0.37[a]	0.118	0.005
Fecal excretion, *% of intake*	1.00	1.30	1.15	1.13	0.138	0.478
Urinary excretion, *% of intake*	3.29	2.83	3.60	3.25	0.853	0.945
Retention, *g/d*	14.7[a]	12.5[b]	12.0[b]	12.8[b]	1.13	0.010
Retention, *% of intake*	95.7	95.9	95.3	95.6	0.943	0.974
Mg						
Intake, *g/d*	0.61[a]	0.58[ab]	0.54[b]	0.49[c]	0.050	0.001
Ileal flow, *% of intake*	1.79	1.99	2.02	3.32	0.548	0.237
Post-ileal flux, *% of intake*	0.60	0.65	1.03	2.47	0.584	0.126
Fecal excretion, *% of intake*	1.19	1.34	0.99	0.85	0.113	0.067
Urinary excretion, *% of intake*	8.30	11.0	17.0	16.8	3.145	0.192
Retention, *g/d*	0.56[a]	0.49[ab]	0.44[bc]	0.39[c]	0.044	0.003
Retention, *% of intake*	90.5	87.7	82.0	82.4	3.17	0.218
P						
Intake, *g/d*	6.59[a]	4.30[c]	4.99[b]	5.32[b]	0.756	0.004
Ileal flow, *% of intake*	1.62[b]	1.80[b]	2.95[b]	6.38[a]	0.897	0.016
Post-ileal flux, *% of intake*	1.17[b]	1.08[b]	2.16[b]	5.20[a]	0.834	0.035
Fecal excretion, *% of intake*	0.46[b]	0.72[b]	0.79[b]	1.18[a]	0.114	0.015
Urinary excretion, *% of intake*	1.28	3.83	4.21	4.25	1.208	0.111
Retention, *g/d*	6.48[a]	4.11[c]	4.74[bc]	5.03[b]	0.296	< 0.001
Retention, *% of intake*	98.3	95.6	95.0	94.6	1.733	0.079

[1] Values are least-squares means; $n = 8$. Means without a common letter differ, $P < 0.05$.

Table 6.3 Intake, ileal flow, postileal flux, fecal excretion, urinary excretion and apparent retention of Na, K, and N in pigs fed 4 starch diets[1]

Diet	S1	S2	S3	S4	Pooled SEM	*P* value
Na						
Intake, *g/d*	3.70	3.74	3.64	3.30	0.309	0.062
Ileal flow, *% of intake*	1.22^{c}	1.36^{c}	2.31^{b}	3.25^{a}	0.249	< 0.001
Post-ileal flux, *% of intake*	1.15^{c}	1.24^{c}	2.21^{b}	3.16^{a}	0.261	< 0.001
Fecal excretion, *% of intake*	0.07	0.12	0.10	0.08	0.023	0.559
Urinary excretion, *% of intake*	32.8	49.6	37.4	30.6	6.38	0.151
Retention, *g/d*	2.45	1.86	2.20	2.23	0.204	0.195
Retention, *% of intake*	67.1	50.3	62.5	69.4	6.38	0.150
K						
Intake, *g/d*	3.60^{c}	4.65^{bc}	4.72^{ab}	4.82^{a}	0.401	0.002
Ileal flow, *% of intake*	0.21	0.15	0.43	0.63	0.124	0.078
Post-ileal flux, *% of intake*	0.09	0.05	0.27	0.35	0.118	0.261
Fecal excretion, *% of intake*	0.12^{b}	0.09^{b}	0.16^{b}	0.28^{a}	0.028	0.008
Urinary excretion, *% of intake*	32.4	36.1	31.1	23.1	5.14	0.072
Retention, *g/d*	2.37^{c}	2.93^{bc}	3.14^{ab}	3.65^{a}	0.192	0.006
Retention, *% of intake*	67.5	63.8	68.8	76.6	5.14	0.076
N						
Intake, *g/d*	39.3	41.7	42.1	43.3	3.64	0.26
Ileal flow, *% of intake*	7.10^{a}	6.34^{ab}	5.64^{b}	3.94^{c}	0.40	0.002
Post-ileal flux, *% of intake*	-1.03^{a}	-0.84^{a}	-1.94^{b}	-8.75^{c}	0.30	< 0.001
Fecal excretion, *% of intake*	8.13^{b}	7.18^{b}	7.58^{b}	12.69^{a}	0.45	< 0.001
Urinary excretion, *% of intake*	24.2	26.9	29.4	31.1	4.90	0.527
Retention, *g/d*	26.0	27.1	25.9	24.4	2.10	0.771
Retention, *% of intake*	67.6^{a}	65.9^{ab}	63.0^{ab}	56.3^{b}	4.85	0.046

[1] Values are least-squares means; $n = 8$. Means without a common letter differ, $P < 0.05$.

Table 6.4 Intake, ileal flow, post-ileal flux, fecal excretion, urinary excretion and apparent retention of Zn, Fe, Cu, and Mn in pigs fed 4 starch diets[1]

Diet	S1	S2	S3	S4	Pooled SEM	*P* value
Zn						
Intake, *mg/d*	199[b]	208[b]	206[b]	236[a]	18.6	0.019
Ileal flow, *% of intake*	1.86	1.81	1.87	2.32	0.457	0.836
Post-ileal flux, *% of intake*	-0.84	-0.71	-0.72	0.69	0.395	0.098
Fecal excretion, *% of intake*	2.70[a]	2.51[a]	2.59[a]	1.63[b]	0.208	0.010
Urinary excretion, *% of intake*	6.93	8.93	10.50	7.06	2.301	0.634
Retention, *mg/d*	180	185	181	217	20.9	0.081
Retention, *% of intake*	90.4	88.6	86.9	91.3	2.43	0.561
Fe						
Intake, *mg/d*	318[a]	260[b]	325[a]	232[b]	24.5	< 0.001
Ileal flow, *% of intake*	1.70[b]	2.03[ab]	1.77[b]	3.82[a]	0.594	0.048
Post-ileal flux, *% of intake*	-0.52	-0.74	-0.52	0.91	0.56	0.172
Fecal excretion, *% of intake*	2.22[b]	2.77[a]	2.29[b]	2.90[a]	0.088	0.002
Urinary excretion, *% of intake*	2.43	3.78	3.99	3.71	1.303	0.846
Retention, *mg/d*	303[a]	243[b]	305[a]	218[b]	24.6	< 0.001
Retention, *% of intake*	95.4	93.5	93.7	93.4	1.34	0.730
Cu						
Intake, *mg/d*	86.6[a]	53.2[b]	92.1[a]	63.3[b]	6.51	< 0.001
Ileal flow, *% of intake*	1.49	2.51	1.45	3.06	0.572	0.189
Post-ileal flux, *% of intake*	-0.41	-0.63	-0.35	0.80	0.564	0.310
Fecal excretion, *% of intake*	1.89[c]	3.14[a]	1.79[c]	2.26[b]	0.057	< 0.001
Urinary excretion, *% of intake*	2.61	5.79	4.76	3.79	1.421	0.540
Retention, *mg/d*	82.7[a]	48.6[c]	86.2[a]	59.8[b]	6.50	< 0.001
Retention, *% of intake*	95.5	91.1	93.4	94.0	1.45	0.314
Mn						
Intake, *mg/d*	54.6[bc]	78.2[a]	50.2[c]	59.7[b]	5.31	< 0.001
Ileal flow, *% of intake*	1.63	1.26	1.95	2.34	0.382	0.277
Post-ileal flux, *% of intake*	-0.72[b]	-0.46[ab]	-0.57[ab]	0.46[a]	0.380	0.049
Fecal excretion, *% of intake*	2.34[b]	1.72[c]	2.52[a]	1.88[c]	0.049	< 0.001
Urinary excretion, *% of intake*	2.70	2.53	5.55	3.35	1.476	0.543
Retention, *mg/d*	51.9[bc]	74.9[a]	46.2[c]	56.8[b]	5.42	< 0.001
Retention, *% of intake*	95.0	95.8	91.9	94.8	1.50	0.410

[1] Values are least-squares means; $n = 8$. Means without a common letter differ, $P < 0.05$.

6.3.2 Mineral and N balance

Ileal flow, post-ileal flux, and fecal excretion of macro- and trace minerals and N were affected ($P < 0.05$) by different starch diets. Ileal flow of Ca, Na, K, Zn, Fe, and N; post-ileal flux of Ca, Na, Mn, and N; and fecal excretion of K, Fe, Cu, and N was higher ($P < 0.05$) in pigs fed the S4 diet compared to pigs fed the S1 diet. Starch diets did not affect urinary excretion of macro- and trace minerals and N in pigs fed different starch diets. Body retention (g/d basis) of macro- and trace minerals followed the pattern of daily intake of respective minerals. Body retention (% of intake basis) of macro- and trace minerals did not differ among pigs fed different 4 starch diet except P retention, (% of intake) was lower ($P < 0.05$) in pigs fed the S4 diet compared to pigs fed S2 diet. Body retention (% of intake basis) of N was lower ($P < 0.05$) in pigs fed the S4 diet compared to when they were fed the S1 diet (**Table 6.2, 6.3 and 6.4**).

6.3.3 Interrelation among starch characteristics and post-ileal profile of minerals, bacteria, and SCFA

Ileal flow of macro minerals had linear positive relation ($P < 0.01$) with ileal flow of starch (**Figure 6.1**). Principal component analysis showed that amylose content was positively related with ileal flow of starch, P, K, Na, Ca and phytate content of diet and negatively related with ileal flow of N. Phytate content was positively related with ileal flow of macro- and trace minerals (**Figure 6.2**). Similarly, post-ileal flux of minerals was positively related with amylose content, fecal total and individual SCFA, post-ileal starch flow, *Lactobacillus* group, and *Bifidobacterium*

spp. and has poor relation with total bacteria, *Enterobacteriaceae*, Bacteroides-Prevotella-Porphyrmonas, and *Enterococus* spp. Phytate content was positively related with population of major bacterial groups such as Bacteroides-Prevotella-Porphyrmonas, *Cl.* clusters I, IV and XIVa, and total bacteria (**Figure 6.3**). In vitro starch digestion rate was positively related with retention of P, N, and Mg (**Figure 6.4**). Phytate content in diet was positively related with amylose content and retention of Ca, Mg, Zn, Mn, Cu, and Fe and negatively related with retention of P and N. Regression analysis showed that post-ileal Mg flux had positive linear relationship ($P < 0.01$) with fecal propionate ($R^2 = 0.35$), butyrate ($R^2 = 0.30$), and total SCFA ($R^2 = 0.34$) (**Figure 6.5**). Post-ileal Na flux had linear positive relationship ($P < 0.01$) with fecal acetate ($R^2 = 0.55$), propionate ($R^2 = 0.62$), butyrate ($R^2 = 0.45$), and total SCFA ($R^2 = 0.64$) (**Figure 6.6**). In addition, post-ileal N flux has linear negative relationship ($P < 0.01$) with fecal acetate ($R^2 = 0.64$), propionate ($R^2 = 0.54$), butyrate ($R^2 = 0.50$), total SCFA ($R^2 = 0.63$), and *Bifidobacterium* spp. ($R^2 = 0.27$) (**Figure 6.7**). Post-ileal flux of Fe had linear positive relationship ($P < 0.01$) with fecal butyrate content ($R^2 = 0.25$) (**Figure 6.8**).

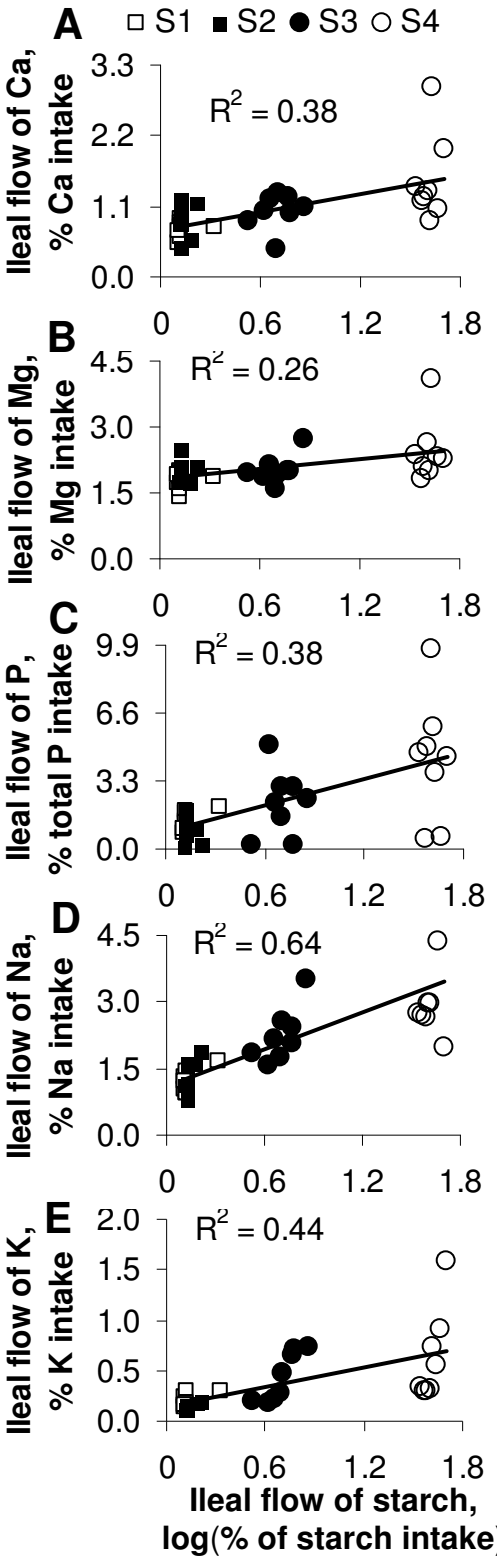

Figure 6.1 Linear relation ($P < 0.01$) of ileal starch flow with ileal flow of Ca (**A**), Mg (**B**), P (**C**), Na (**D**), and K (**E**) in pigs consuming 4 starch diets differing in rate of digestion; ; values are means; $n = 8$.

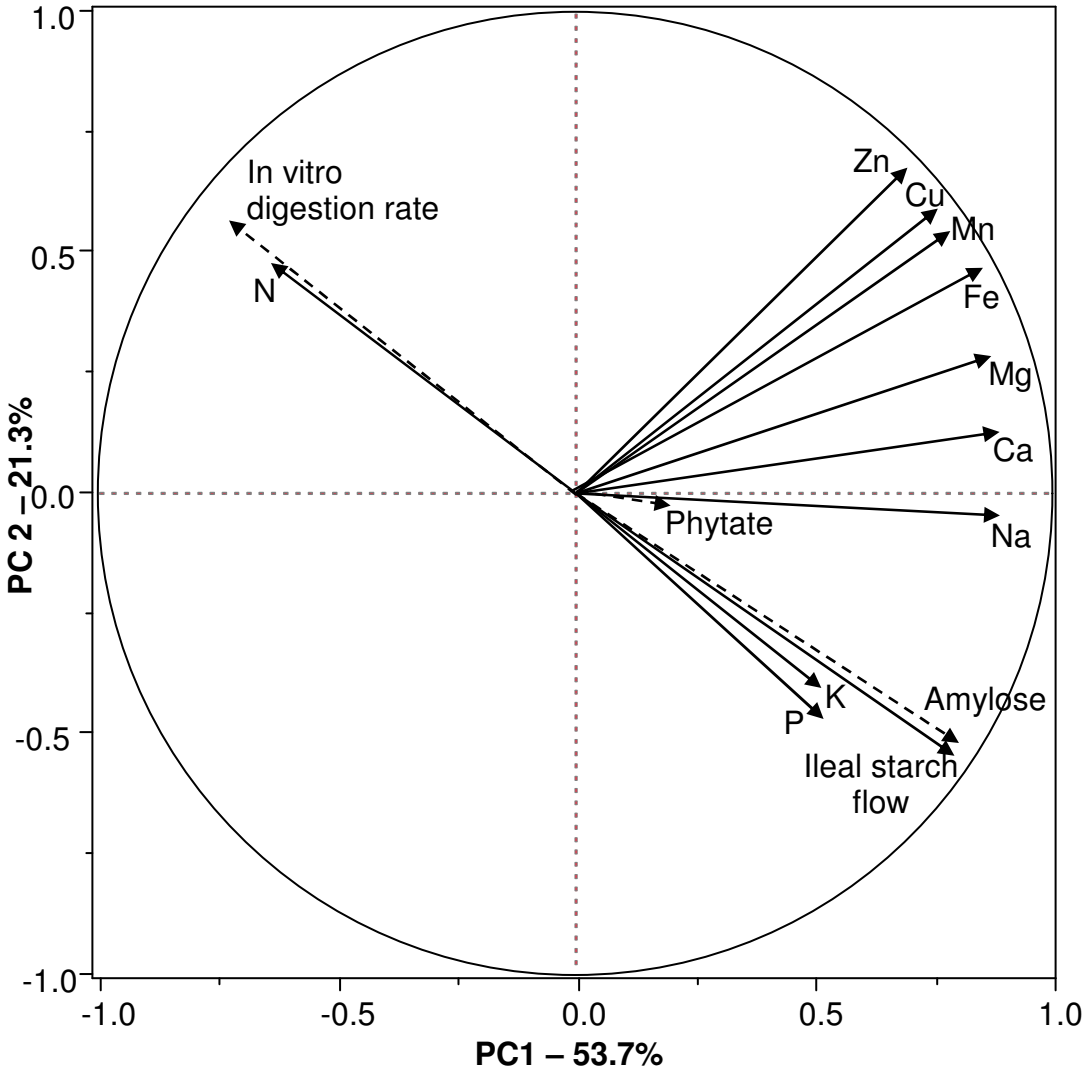

Figure 6.2 Loading plot showing the correlations among starch characteristics, phytate content in diet (dashed arrows), and ileal flow of starch and macro- and trace minerals (solid arrows) of the first two eigenvalues (PC 1 and PC 2) in pigs consuming 4 starch diets differing in the rate of digestion; values except the starch characteristics are based on % of intake; n = 8.

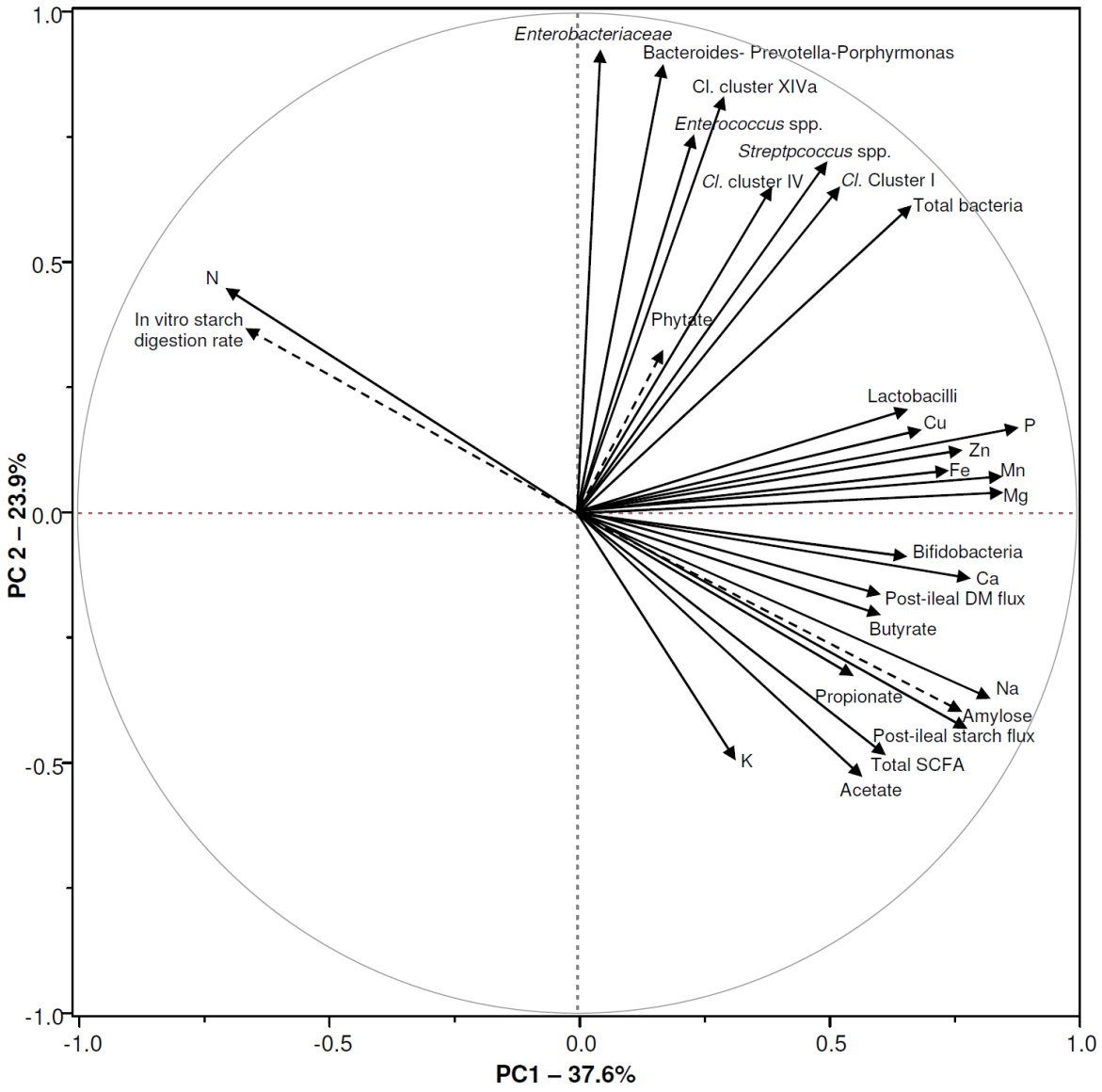

Figure 6.3 Loading plot showing the correlations among starch characteristics, phytate content in diet (dashed arrows), post-ileal DM and starch disappearance, post-ileal flux of macro- and trace minerals and N, fecal SCFA, and fecal bacterial population (solid arrows) of the first two eigenvalues (PC 1 and PC 2) in pigs consuming 4 starch diets differing in the rate of digestion; values except the starch characteristics are based on % of intake; n = 8.

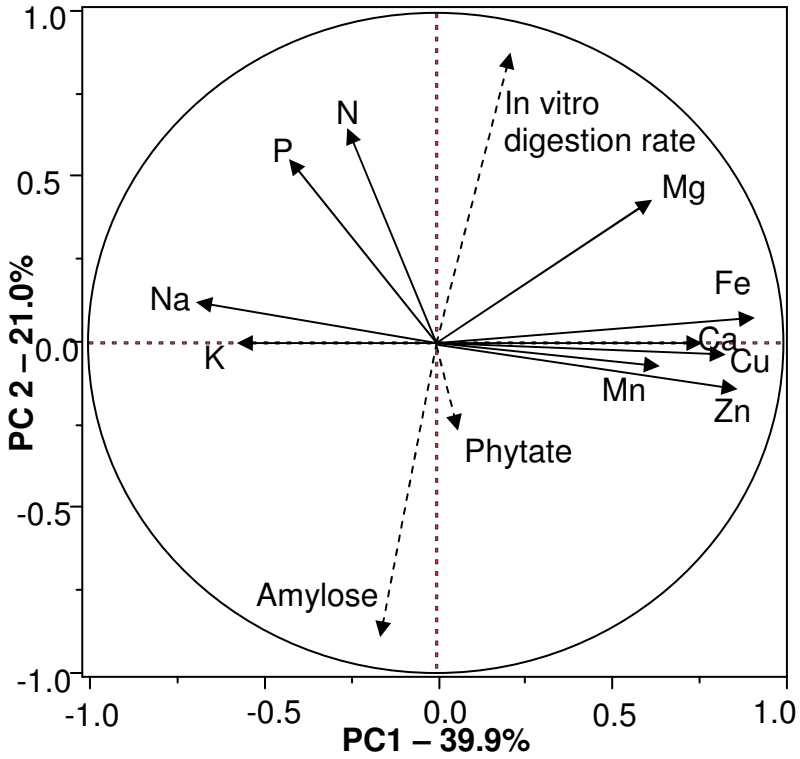

Figure 6.4 Loading plot showing the correlations among starch characteristics, phytate content in diet (dashed arrows), and body retention of macro- and trace minerals (solid arrows) of the first two eigenvalues (PC 1 and PC 2) in pigs consuming 4 starch diets differing in the rate of digestion; values except the starch characteristics are based on % of intake; n = 8.

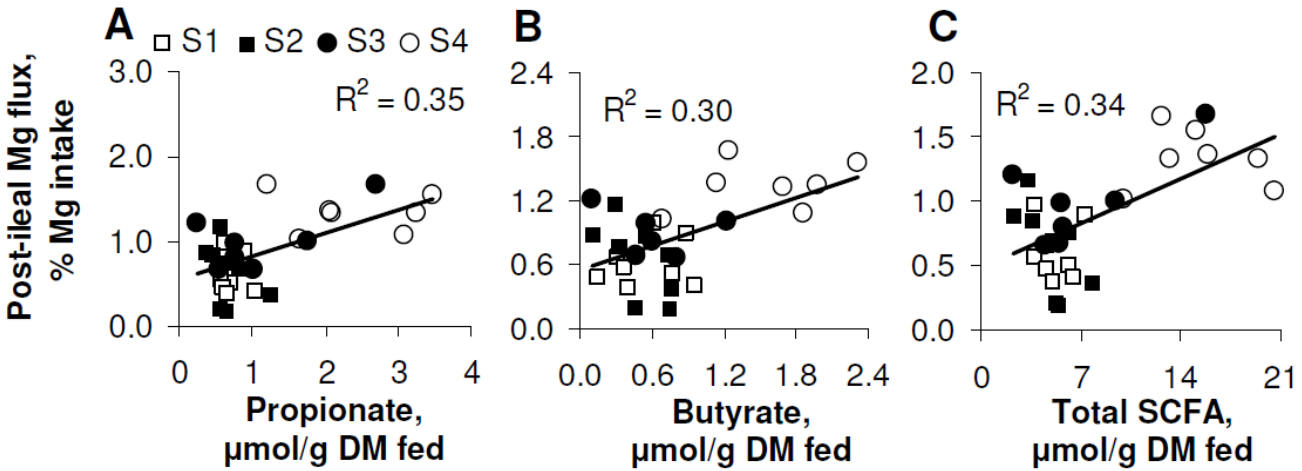

Figure 6.5 Linear positive relation ($P < 0.01$) of post-ileal flux of Mg with propionate (**A**), butyrate, (**B**), and total SCFA (**C**) in pigs consuming 4 starch diets differing in rate of digestion; $n = 8$.

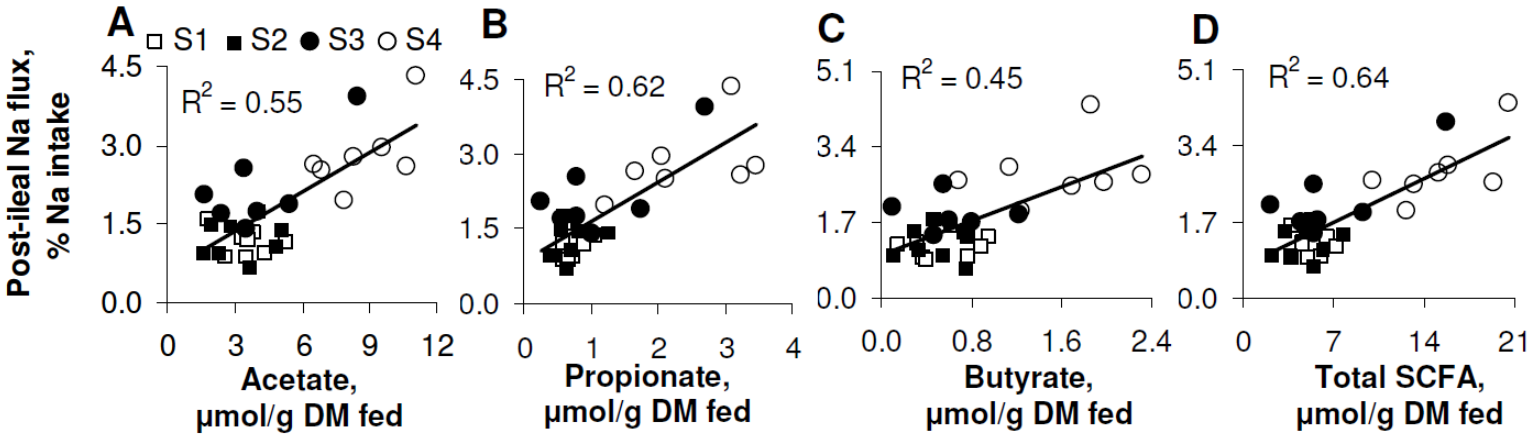

Figure 6.6 Linear positive relation ($P < 0.01$) of post-ileal flux of Na with acetate (A), propionate (B), butyrate (C), and total SCFA (D) in pigs consuming 4 starch diets differing in rate of digestion; n = 8.

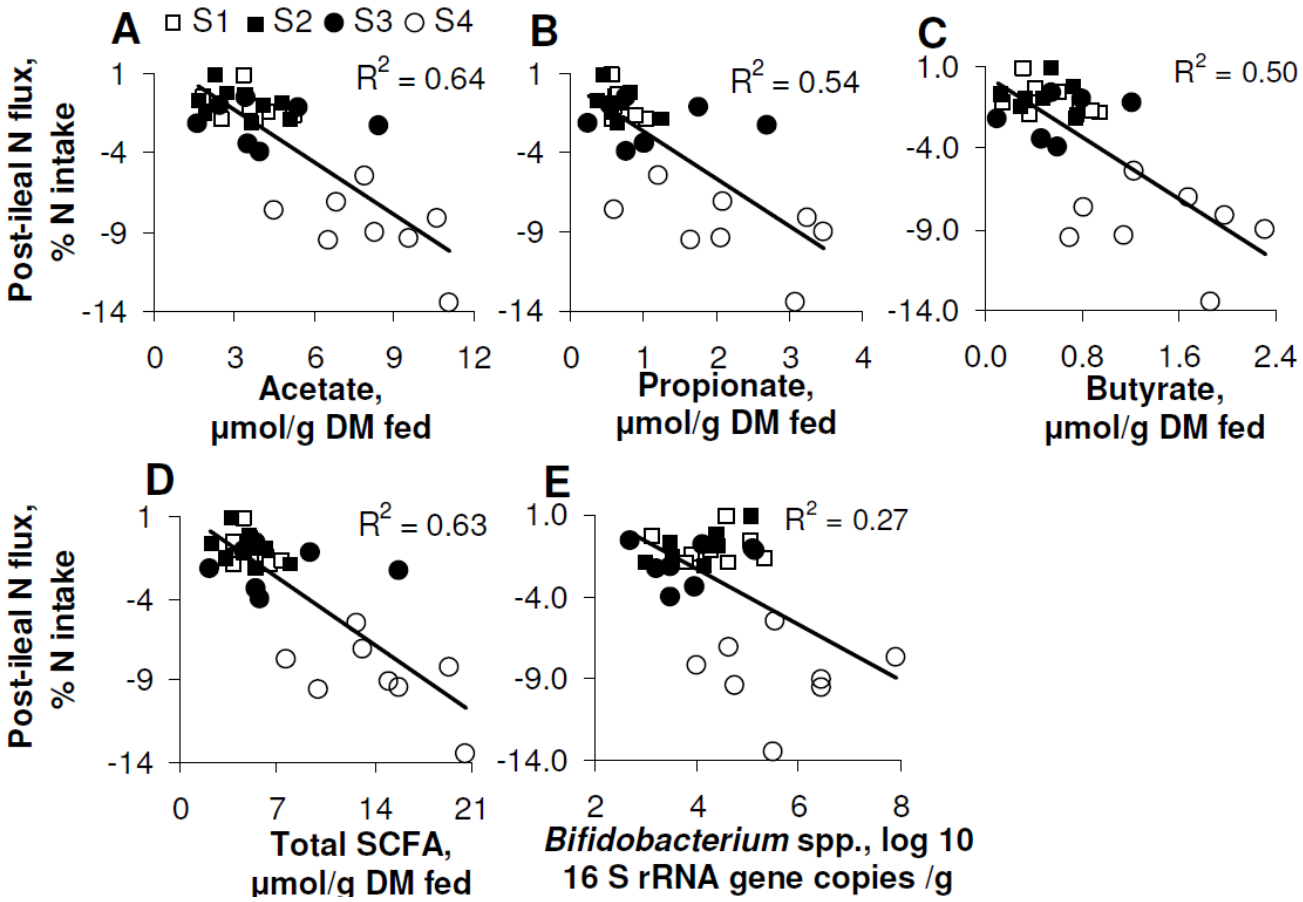

Figure 6.7 Linear negative relation ($P < 0.01$) of post-ileal flux of N with acetate (A), propionate (B), butyrate (C), and total SCFA (D), and Bifidobacterium spp. (E) in pigs consuming 4 starch diets differing in rate of digestion; n = 8.

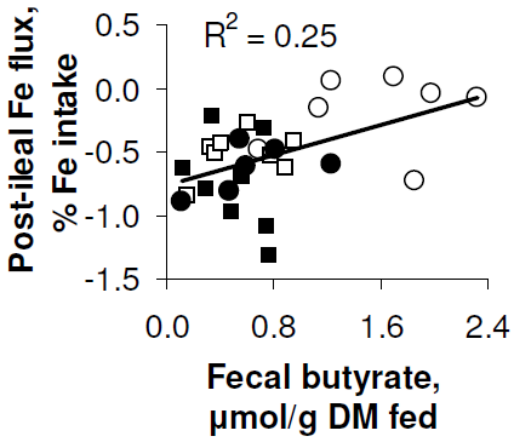

Figure 6.8 Linear negative relation ($P < 0.01$) of post-ileal flux of Fe with fecal butyrate in pigs consuming 4 starch diets differing in rate of digestion; n = 8.

6.4 Discussion

The role of starch on nutritional physiology has been studied over many years; however, only a few studies reported the effect of starch types on the mineral digestion and metabolism in monogastrics animals (Bronner et al., 1986; Schulz et al., 1993; Morais et al., 1996; Younes et al., 1996; Bird et al., 2000; Kishida et al., 2001; Lopez et al., 2001). The present study evaluated the effects of purified non-gelatinized starches with a wide range in physicochemical properties on ileal flow, post-ileal flux, and apparent retention of macro- and trace minerals and their interrelation with fermentation characteristics in the gut. The findings of this study showed that starch with high amylose and low in vitro digestibility increases ileal flow and post-ileal flux of macro- and trace minerals which confirmed the study hypothesis.

The present study findings should be carefully interpreted because the mineral content differed among the diets. Pigs fed S4 diet had lower intake of Ca, Mg, Fe,

Cu and higher intake of K and Zn compared to pigs fed S1 diet. The non-starch components were consistent among the 4 diets so that the dissimilar mineral amounts were solely due to the differences in intrinsic mineral profiles of the purified starches. As mineral from inorganic sources have higher digestibility compared to plant-derived minerals (NRC, 1998), compensation for differences in dietary mineral content seemed better than balancing the diets with inorganic mineral sources. Previously, greater dietary intake decreased body retention of minerals (as % of mineral intake) by decreasing intestinal absorption and increasing fecal and urinary excretion (Weigand and Kirchgessner, 1980; Rodehutscord et al., 1999). In the present study, fecal excretion increased in pigs fed S4 diet with higher K content probably indicating confounding effects of dietary level of minerals and starch chemistry on fecal excretion and retention of K. However, such effects were not observed with greater intake of Ca, Mg, Fe, and Cu by pigs fed S1 diet probably indicating much stronger effect of diet on mineral balance compared to sole homeostatic regulation. In the present study, confounding effect of differing dietary mineral was minimized by presenting the data per unit of individual mineral intake rather than per unit of DM intake.

In the present study, daily intakes of Ca and Mg were marginally higher than recommended for growing finishing pig. Daily intake of total P was lower than recommended for growing finishing pig; however, the available P was sufficient to meet the P recommendations (NRC, 1998). The present study findings showed that more than 93% of the total dietary intake of macro- and trace minerals was

absorbed in the small intestine which supported the current concept that small intestine is indeed the major site of mineral absorption in swine. Ileal flow of minerals were consistently (either significantly, for Ca, Na, K, Zn, Fe, or numerically, for remaining minerals) higher in pigs fed S4 diet compared to other 3 diets. Among the minerals P had the highest ileal flow (6.38%), followed by Fe (3.82%), Mg (3.32%), Na (3.25%), Cu (3.06%), Mn (2.34%), Zn (2.32%), Ca (1.51%), and K (0.63%). In addition, ileal starch flow had strong positive relationship with ileal flow of macro-minerals indicating small intestinal mineral digestion and absorption positively contributes to enzymatic starch digestibility in monogastrics species. Although, direct comparison could not be made due to differences in pigs' age group, the digestibility of macro- and trace minerals in this study was higher than previously reported with semipurified diets in young pigs (Woyengo et al., 2009). Inclusion of marginally sufficient amount of minerals in purified diet to meet the pigs' requirements (NRC, 1998) could be the reason for higher mineral digestibility in the present study.

In addition to mineral absorption in small intestine, its digestion and absorption can take place in the large intestine (Hardwick et al., 1990). The role of large intestine in mineral absorption is particularly important during higher mineral intake or a less efficient absorption in the small intestine. In the present study, post-ileal flux of minerals was consistently (either significantly, for Ca, Na, Mn, or numerically, for remaining minerals) higher in pigs fed S4 diet compared to the remaining 3 diets. In addition, post-ileal flux of macro- and trace minerals

particularly, Ca, Mg, Na, K, and Fe were positively related with fermentation products i.e., SCFA in feces. This finding is supported by previous observations in rats and pigs where highly fermentable carbohydrates, such as pectin, lactulose, cellulose, and resistant starch, increased the cecal absorption of minerals by stimulating fermentation in the hindgut (Demigne et al., 1989; Metzler-Zebeli et al., 2010). Previously it has been reported that fermentation of carbohydrates in the large intestine can release minerals from the undigested starch fractions; reduced pH from microbial fermentation products increases mineral solubility (Schulz et al., 1993) which favours their absorption from the large intestine. Moreover, SCFA such as propionate has been found to be associated with colonic absorption of macro minerals in humans (Trinidad et al., 1993). Although the role of the large intestine in mineral absorption is assumed to be small in pigs (Schroder and Breves, 2007), the present data indicate that the large intestine may play a major role in mineral retention by compensating small intestinal mineral absorption when low digestible starch or NSP fractions are included in the diet that interfere with cation absorption in the small intestine.

As distal intestinal bacteria have their own requirements (Durand and Komisarczuk, 1988; Groot et al., 2005), they may compete with the host for macro- and trace minerals. The minerals used by bacteria for their growth will likely be excreted in feces. The post-ileal net secretion of Ca, Zn, Fe, and Cu in pigs fed S1, S2 and S3 diets may be due to excessive dietary intake or can be due to large intestinal bacterial growth. In the present study, post-ileal flux of macro-

and trace mineral had strong positive correlation with fecal Bifidobacteria and Lactobacilli group and had poor correlation with fecal Enterobacteriaceae, Bacteroides-Prevotella Porphyrmonas, and *Closstridium* clusters. This finding may suggest that the beneficial microorganism likely play a role in digestion and absorption of minerals in large intestine in monogastric species.

In the present study, body mineral retention was not affected by starch types. This is due to greater post-ileal flux compensated the greater ileal flow in pigs fed S4 diet compared to the remaining 3 diets. Previously, overall gut absorption of Ca was either increased (Schulz et al., 1993; Morais et al., 1996) or not affected (Kishida et al., 2001) by slowly digestible starch. In addition, retention of Ca (Younes et al., 1996) and Mg (Younes et al., 1996; Lopez et al., 2001) was increased but retention of P (Morais et al., 1996; Kishida et al., 2001) was not affected by diets containing slowly digestible starch. The absorption of Fe is increased and that of Zn was not affected (Younes et al., 1996) by slowly digestible starch (Morais et al., 1996; Kishida et al., 2001). In addition retention of Ca, Mg, Zn, Fe, and Cu was increased by slow release starch compared to rapid release starch (Lopez et al., 2001). Hence, these findings clearly indicate that slowly digestible starch may improve mineral absorption via increasing solubility of minerals in the large intestine. However, being an anion, the mechanism of intestinal absorption of P is much different than other minerals which are cations. Further study is needed to clarify mechanism behind greater P absoption in the large intestine.

Nitrogen is present in protein and non-protein N components in feed. In the present study, pigs fed slowly digestible starch had lower ileal flow of N but had higher net large intestinal secretion and fecal excretion of N; thus resulting lower net retention of N compared to pigs fed the remaining 3 diets. Since there was no effect of diets on N excretion in urine, hindgut N loss likely reflects mucus, epithelium etc. (endogenous protein losses) rather than Urea flow from blood. In addition, post-ileal N flux had negative relationship with fecal contents of total and individual SCFA and gene copies of bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp. These findings clearly indicate the higher N utilization for microbial growth and fermentation in the large intestine that probably resulted in greater loss of N in feces as bacterial N in pigs fed slowly digestible starch diet (Chapter 5).

Phytate is mainly present in the bran (aleurone layer, testa and pericarp) of rice grain, in the germ of corn seed and cotyledon of pea seed (Baker, 1991). Contamination of phytate from these sources during starch purification could be the source of phytate in starch sources S2 and S4. Phytate can bind with minerals such as Ca, Mg, Zn, Cu, Co, Mn, and Fe. This binding potentially renders these minerals unavailable for intestinal absorption (Maddaiah et al., 1964; Vohra et al., 1965). However, in the present study, such effect was not observed because phytate did not have negative relation with both ileal flow and post-ileal flux of macro- and micro minerals. Based on PCA analyses, starch characteristics such as

amylose and in vitro rate digestibility had greater role in small and large intestine mineral digestibility than phytate. Further study is needed to understand the interrelation of dietary phytate and starch characteristics on mineral digestibility in monogastric species.

In conclusion, ileal flow of macro- and trace minerals has strong relation with ileal starch flow and thus, can be major determinator of microbial fermentation in the large intestinal. Although mineral absorption in the large intestine is proportionately very small, large intestinal mineral flux can compensate lower mineral absorption in small intestine in pigs fed slowly digestible starch.

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Chapter 7 General Discussion

7.1 Summary and Conclusions

Starch typically constitutes a major portion of diets for humans and monogastric animals. Starch characteristics such as amylose and RS contents are good indicators of in vivo response; however, these characteristics alone can not sufficiently explain starch effects in vivo (Panlasigui et al., 1991). Starch digestion in the small intestine is not solely affected by a single, distinct chemical entity but by a combination of physical and chemical characteristics. Therefore, a need exists to find an accurate starch variable to describe starch responses in vivo. In Chapter 2, the rate of in vitro starch digestibility corrected for gastric emptying described up to 95% of the variability of glucose absorption in pigs. Hence, the rate of in vitro starch digestibility at least up to 4 h can be considered as a starch characteristic that will consistently predict effects of starch on in vivo responses. Therefore, the rate of in vitro starch digestion was used as a link to determine the effect of starch chemistry on in vivo responses in subsequent chapters in the thesis. Furthermore, chapter 2 findings clearly showed that widely used term describing starch fraction such as RDS, SDS, and RS have less biological relevance and are thus of less value for predicting the in vivo responses to starch sources.

The kinetics of starch digestion influence postprandial glucose and insulin responses which are associated with the risk of metabolic diseases such as type II diabetes and obesity in humans (Mathers and Daly, 1998). In addition, luminal

glucose and SCFA that are consequences of starch digestion and fermentation, stimulate the secretion of incretins such as GIP and GLP-1 (Baggio and Drucker, 2007). Increased blood glucose concentration stimulates insulin secretion, and incretins potentiate glucose-stimulated insulin secretion (Baggio and Drucker, 2007). Although amylose (Behall and Hallfrisch, 2002; Deng et al., 2009) and RS (Raben et al., 1994; van der Meulen et al., 1997; Deng et al., 2009) affects peripheral glucose and insulin responses, the association that allows starch chemistry to affect the absorption of starch-derived nutrients and secretion of insulin and incretins is not clear. In addition, the relationship between the kinetics of nutrient absorption and hormone secretion is poorly explained. Chapter 3 updated our current understanding of starch effects on the kinetics of nutrient absorption and insulin and incretin absorption. The findings showed that starch with higher amylose content and lower rate of in vitro digestion decreased glucose absorption and insulin secretion and increased SCFA absorption. The study also revealed that in vivo starch effects can be better described by combining in vitro starch digestion with other physicochemical characteristics of starch. Glucose absorption is an important modulator of insulin secretion; however, other physiological mechanisms such as chemo-sensing in the digestive tract must exist to explain the insulin secretion peaking prior to peak glucose uptake into the portal vein.

Nuclear magnetic resonance (NMR) is increasingly used for metabolite profiling of body fluids such as plasma to understand the role of nutrition on metabolic

physiology in animals (Bertram et al., 2009; Lankinen et al., 2009). Recently, NMR is used in studies involving dietary intervention in monogastric species such as swine. Chapter 4 updated our understanding on plasma biomarkers that are influenced by dietary starch. The findings showed that the NMR technique can be used to understand the qualitative effects of changes in dietary treatments on the kinetics of profile of metabolites in portal and arterial plasma in swine. Starch chemistry affected the profile of NMR based metabolites in portal and arterial plasma samples. Starch with high amylose and low in vitro digestibility was energetically less efficient, promoted use of essential amino acids by enterocytes and PDV thus lowering body use of the amino acids, and increased plasma betaine concentration potentially beneficial for human health.

Previously, diets containing starch with higher content of amylose and RS increased digesta mass, SCFA production, and population of *Bifidobacterium* spp. and Lactobacilli group in the gut (Brown et al., 1997; Kleessen et al., 1997; Wang et al., 2002; Morita et al., 2004; Bird et al., 2007). Although diets with high amylose and RS content decrease nutrient digestibility and increase gut microbial fermentation (Higgins, 2004; Englyst and Englyst, 2005; Singh et al., 2010), the relationship among starch characteristics, nutrient flow and microbial activity and profile is not clearly understood. Chapter 5 findings showed that starch with higher amylose and lower *in vitro* digestibility increased post-ileal nutrient flow and microbial fermentation, and selectively promoted *Bifidobacterium* spp. in the distal gut. The study further demonstrated that substantial changes of starch

chemistry (at least ≥40% amylose content and ≤0.36%/min of maximum in vitro digestion rate) were required to have potentially beneficial changes in the post-ileal variables in pigs.

Diets containing different starch types may affect the solubility and bacterial utilization of minerals and absorptive surface area of gut epithelium (Schulz et al., 1993; Younes et al., 1996; Hrdina et al., 2009) and thus, may modulate absorption and body retention of minerals (Abrams et al., 2007; Demigne et al., 2008). Thus, a need exists to define the role of starch chemistry on mineral absorption in the small intestine and to elucidate the consequences of starch fermentation on mineral absorption in the large intestine of monogastric species. In Chapter 6, ileal starch flow strongly influences the ileal flow of macro- and trace minerals. Although mineral absorption in the large intestine is proportionately very small in high starch diet, large intestinal mineral flux probably facilitated by higher fermentation can compensate lower mineral absorption in small intestine in slowly digestible starch. In addition, greater large intestinal fermentation results in lower N retention in pigs fed slowly digestible starch diet.

7.2 Challenges and Limitations

The research study clarified the role of purified starches differing in amylose content and rates of in vitro digestibility on kinetics of nutrient absorption, hormone release, gut nutrient flow, and fermentation profile. However, challenges

existed throughout the study. The porto-arterial catheterization model is limited in experimental units due to complexity of surgical procedure. The measurements obtained from such a model have a higher variability than other surgical models (Bach Knudsen, 1992). In addition, both the ileal-cannulated and porto-arterial catheterization models provide only the end-point of processes, but not details about the dynamic process of complete digestion and absorption. Finally, the feeding of powdery semi-purified high diets was the challenge, because more time was required to adapt the pigs that are generally fed coarse cereal-based diets.

7.3 Future Research

Starch and its role in digestive physiology has been the subject of research over many years. However, studies explaining the mechanisms of physiological role of starch are rare. The present study revealed several important aspects of starch effects on in vivo responses. However, several other aspects require clarification.

In the present thesis, insulin release peaked before peak glucose absorption. Mechanism of chemo-sensing in the digestive tract must be understood in depth to clarify the findings. Furthermore, slowly digestible starch could be a potential dietary component that may increase release of GLP-1, a potential therapy for type II diabetes (Holst et al., 2009). Studies using the same treatment arrangement using slaughter model may explain the mechanism behind effects on GLP-1 and insulin release. Approaches such as gene expression and immuno-histochemistry

of enteroendocrine cells and β-cells of pancreas will help explain the exact site and effects of slowly digestible starch.

The complex interactions of gut microbiota and host mucosa, which are key to gut health, are influenced by various components of diets that require further detailed exploration. Not only requires gut microbiology further exploration, but also the metabolic pathways used by gut microflora and their effect on host physiology should be understood. The bifidobacteria that are selectively promoted by slowly digestible starch needs to be characterized and species needs to be identified by using nutrigenomic approaches.

In addition, the approaches used in the present study with healthy pig models can be applied with diseased animal models. The findings from such a study might be more relevant to the mechanisms involved in humans with metabolic syndromes such as type II diabetes. Use of unprocessed starch, which is not the case in human nutrition, in the present study may limit direct application of present study findings in humans, interrelationship between processing and starch characteristics such as amylose and in vitro rate of digestion should be explored for direct application of present study findigs in human nutrition. Finally, a need exists to understand the role of purified starch on the metabolism of fatty acids and triglycerides which will be relevant in understanding the mechanism related to metabolic syndrome such as obesity and cardiovascular diseases.

7.4 References

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