The role of starch and fiber composition on nutrient flow, site and extent of nutrient digestion, and microbial profiles in pigs

By

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ABSTRACT

Starch is the main energy substrate for monogastrics, including swine. Physico-chemical properties of starch and the cereal grain matrix can change the kinetics of digestion. Objectives of this thesis were to enhance the understanding of how the physico-chemical properties of purified starch and starch and fiber from whole grains can alter the site and extent of nutrient digestion, microbial and metabolite profiles, and efficiency of energy utilization.

In study 1, weaned pigs (n = 32) were fed one of four diets containing 67% starch with 0, 20, 28, or 63% amylose for 21 d. Increasing dietary amylose linearly increased post-ileal flow of dry matter and starch, hindgut fermentation, and cecal and colonic digesta *Bifidobacterium* spp. However, increasing levels of dietary amylose linearly reduced feed intake and growth indicating high amylose may be less energy efficient than rapidly digestible starch. In studies 2 and 3, 5 whole grains: (% amylose, β-glucan, total dietary fiber (TDF)): 1) high-fermentable, high β-glucan hull-less barley (HFB) (0, 10, 22); 2) high-fermentable, high amylose hull-less barley (HFA) (18, 7, 18); 3) moderate-fermentable hull-less barley (MFB) (11, 5, 15); 4) low fermentable hulled barley (LFB) (11, 4, 17); and 5) low fermentable hard red spring wheat (LFW) (12, 1, 14) were fed to ileal cannulated pigs (n = 7) in a 6 (periods) × 7 (diets) Youden square design. Fermentability of grains was based on the β-glucan content. Highly fermentable whole grains, HFB and HFA, had decreased ileal digestibility of dry matter, starch, and amino acids. However HFB and HFA had increased hindgut substrate availability and starch and DM fermentation. Increased ileal flow of starch from HFB and HFA was

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positively correlated to a shift in fecal microbial profile towards Firmicutes, including genera *Dialister* and *Sharpea*. In study 4, weaned pigs (n = 6/treatment) were fed one of six diets in a 3 (wheat (W) (%amylose, %β-glucan, %TDF) (12, 1, 14), digestible hull-less barley (dHB) (11, 5, 15), and fermentable hull-less barley (fHB) (18, 7, 18) × 2 (SBM or autoclaved soybean meal (aSBM)) factorial arrangement for 18 days. Higher viscosity of dHB and fHB increased post-ileal β-glucan flow, providing a fermentative substrate to the hindgut. Increased hindgut substrate availability from hull-less barley shifted the colonic microbiota towards Firmicutes and Actinobacteria and increased total tract nutrient digestibility and piglet feed efficiency.

The findings from this thesis provide evidence that physico-chemical properties of starch and fiber are important determinants of digestion kinetics. Dietary inclusion of high amylose starch or fermentable fiber has shown to increase hindgut substrate availability and shift microbial and metabolite profiles. Thus amylose or cereal grains high in fermentable fiber may be beneficial for dietary inclusion if careful consideration of efficiency of energy utilization and amino acid digestibility is taken to ensure adequate pig growth performance.

PREFACE

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AA	Amino acid
ADF	Acid detergent fiber
ADFI	Average daily feed intake
AOAC	American association of cereal chemists
aSBM	Autoclaved soybean meal
ATTD	Apparent total tract digestibility
AID	Apparent ileal digestibility
BCFA	Branched chain fatty acid
BW	Body weight
CCAC	Canadian council on animal care
CP	Crude protein
DE	Digestible energy
dHB	Digestible hull-less barley
DM	Dry matter
DMI	Dry matter intake
fHB	Fermentable hull-less barley
FHP	Fasting heat production
GC	Gas chromatography
GE	Gross energy
GIT	Gastrointestinal tract
HFA	High-fermentable, high amylose hull-less barley
HFB	High-fermentable, high β-glucan hull-less barley
LFB	Low fermentable hulled barley
LFW	Low fermentable hard red spring wheat
ME	Metabolizable energy
MFB	Moderate-fermentable hull-less barley
N	Nitrogen
NDF	Neutral detergent fiber
NE	Net energy
N-free	Nitrogen free

NSP	Non-starch polysaccharides
PCA	Principle component analysis
qPCR	Quantitative polymerase chain reaction
RDS	Rapidly digestible starch fraction
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
SBM	Soybean meal
SCFA	Short chain fatty acids
SEM	Standard error of mean
SDS	Slowly digestible starch
SID	Standardized ileal digestibility
TDF	Total dietary fiber
W	Wheat

Chapter 1. Quantifying site, extent, and kinetics of resistant starch (RS) vs. non-RS digestion and the resulting energy value in pigs

1.1 Introduction

Starch from cereal grains is the main source of energy in swine diets, representing up to 55% of the diet (Knudsen et al., 2006). The rate of starch digestion into single glucose units varies dependent on the chemical composition and processing methods applied to starch (Giuberti et al., 2015). Starch escaping small intestinal digestion, i.e., resistant starch (RS), becomes a substrate for microbial fermentation resulting in production of short chain fatty acids (SCFA) in the hindgut. In swine nutrition, whether dietary RS or digestible starch reduces growth and efficiency due to differences in energetic efficiency of utilization is debated. Rapidly digested starch is thought to be at least 14% more efficient at yielding energy than RS that is fermented into SCFA (Jørgensen et al., 1997). As such, starch varying in rate and site of digestion will influence its energy value and host physiological responses including feed intake, growth, lean and fat deposition, hormonal homeostasis, microbial ecology, and gut health. Thus, proper evaluation of the energy value of starch, especially resistant starch (RS), is needed for accurate diet formulation.

In North America, predictive net energy (NE) equations adapted from Noblet et al., (1994) are conventionally used (NRC, 2012). These predictive equations calculate NE based on measured DE or ME of feedstuffs, for example: NE = (0.700 × DE) + (1.61 × ether extract) + (0.48 × starch) − (0.91 × crude protein) − (0.87 × acid-detergent fiber). These prediction equations have limitations, e.g., the NE value of high fibrous feedstuffs

will be overestimated. Because these equations use total starch content they may also overestimate energy content in high RS feedstuffs. If these prediction equations indeed overestimate energy content of RS-containing feedstuffs, the proper measurement to determine digestibility and energy value of starch remains a question.

Classical swine nutritionists have measured starch digestibility as apparent total tract digestibility (ATTD). However, this method does not differentiate between starch digestion and fermentation and typically results in 100% digestibility (Sun et al., 2006; Cervantes-Pahm et al., 2014). It is of nutritional interest to distinguish site, extent and kinetics of starch digestion; thus, methodologies have been designed and include: use of simple cannulas (Low, 1980), slaughter (Payne et al., 1968), installation of catheters to measure glycemic index (GI) and starch-derived portal vein nutrient fluxes (Rerat et al., 1984a, b), *in vitro* assays mimicking small intestinal digestion (Englyst et al., 1992), and indirect calorimetry to measure energy value. This review will focus on methodologies used to quantify site, extent and kinetics of digestion of RS vs. digested starch in pigs. How these techniques can illustrate differences in digestibility between RS vs. non-RS and consequently energetic efficiency and animal performance will be emphasized.

1.2 Factors influencing rates of starch digestion

1.2.1 Chemical composition

Starch is comprised of amylose and amylopectin, making up 98-99% of dry weight, with the remaining 1-2% being integral lipids in the form of lysophospholipids or free fatty acids (Tester et al., 2004). Both amylose and amylopectin are polymers of glucose consisting of $\alpha - 1,4$ linkages and $\alpha - 1,6$ branches in the case of amylopectin (Figure

1.1) (Topping, 1994; Ao et al., 2007). Classically, starch can be considered waxy, normal or high amylose with <15%, 20-35% and > 40% amylose content, respectively (Tester et al., 2004). Amylopectin has an increased rate of digestion, due to digestive enzymes reaching multiple reducing ends (Copeland et al., 2009). Amylose tends to form insoluble semi-crystalline aggregates during processing and is less digestible (Copeland et al., 2009).

The components of starch amylose and amylopectin are packaged into alternating crystalline and amorphous regions in granules. The amylose and amylopectin containing granules come in a variety of sizes and shapes, dependent on feedstuff source and arrangement of amylose and amylopectin within the crystalline regions (Lindeboom et al., 2004; Copeland et al., 2009). Small granules have a greater digestibility than large granules due to increased surface area (Manelius and Bertoft, 1996; Vasanthan and Bhatty, 1996; Bednar et al., 2001).

Naturally occurring proteins, lipids and fibrous components of cereal grains interact within a cereal grain matrix to impact rate and efficiency of starch metabolism with the matrix components slowing gastric emptying and digestion (Thompson et al., 1984). Lipid–amylose complexes can increase the hydrophobicity of starch granules, impeding digestibility (Vasanthan and Bhatty, 1996). Protein–amylose complexes within the cereal grain matrix also reduce a feedstuffs' SI digestion rate and subsequent GI (Jenkins et al., 1987). Other components including lectins, tannins and phytic acid may inhibit enzymatic degradation in the small intestine further slowing down glucose absorption (Thompson et al., 1984, Jenkins et al., 1987). Lowered glycemic response is correlated

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with the presence of phytic acid, which is explained by a decrease in the rate of digestion (Yoon et al., 1983).

1.2.2 Processing methods

To increase digestibility and feed acceptance of grains to pigs, raw grains are typically processed using technologies such as cracking, grinding, rolling, flaking pelleting, steaming, expanding and extruding. In particular application of heat - processing on starch containing feedstuffs disrupts the crystalline regions and increase α-amylase susceptibility and bioavailability (Bornet, 1993). How starch structure and processing affects digestibility has been described previously (Giuberti et al., 2015).

1.3 Starch Metabolism

1.3.1 Digestion

The nutritive value of starchy feedstuffs is associated with rate, site and extent of digestion. As previously reviewed, digestibility of starchy feedstuffs is dependent on intrinsic and extrinsic factors including botanical origin and processing methods. It is the combined action of endohydrolyases, amylase, and exohydrolases, sucro-isomaltase and maltoglucoamylase that breakdown starch within the SI. Starch hydrolysis begins with an endohydrolyase, salivary α–amylase; however, action of this enzyme is short lived due to timely passage of feed to the stomach. Once in the stomach, HCl secretion by parietal cells increases acid hydrolysis of starch at the expense of salivary α–amylase activity. Although salivary α–amylase plays only a minor role in starch hydrolysis, it is hypothesized to be part of a chemo-sensing mechanism, aiding in the maintenance of hormonal homeostasis (Shirazi-Beechey et al., 2011).

Once in the SI, pancreatic secretions into the duodenum increase pH and restart enzymatic starch hydrolysis with porcine pancreatic α–amylase. Starch hydrolysis by α–amylase might be rate limited due to the intrinsic properties of starch (Slaughter et al., 2001). The α–amylase works in a multiple attack mechanism forming a stable substrate-enzyme complex enabling hydrolysis of multiple bonds (Robyt and French, 1967). The α–amylase has both endo and exohydrolysis action, first hydrolyzing the α–1,4 linkages and subsequently hydrolyzing the newly formed reducing end (Robyt and French, 1970; Koukiekolo et al., 2001). The exo–hydrolysis action of PPA produces short malto–oligosaccharides and α–limit dextrins (MacGregor et al., 2001). Limited studies have assessed action of α–amylase on amylopectin, even though it forms the majority of most native starches. The α–amylase has a low inner chain attack activity on amylopectin, resulting in slower hydrolysis rate (Bijttebier et al., 2010). The finding that α–amylase hydrolysis of amylopectin might be slower than amylose has caused a new theory to emerge that branching density of amylopectin and amylose may be a factor influencing starch digestibility (Ao et al., 2007). Four mucosal enzymes digest α–limit dextrins left by amylase hydrolysis, including N terminus and C terminus subunits of maltoglucoamylase and sucroisomaltase that convert limit dextrins to free glucose. Recently detected, mucosal enzymes may be important to determine starch digestion rate. For example, certain α–limit dextrins were resistant to digestion by α–glucosidases (Lin et al., 2012).

1.3.2 Fermentation

The portion of starch resisting host enzymatic digestion, termed RS in 1982 by Englyst, flows to the distal ileum, cecum and large intestine becoming an ideal substrate for microbial fermentation. Fractions of starch resistant to digestion have been divided into 5 subtypes based on physical and chemical characteristics. For the purpose of this review, RS will refer to RS1, physically entrapped inaccessible starch; RS2, native granular starch; RS3, retrograded starch (Giuberti et al., 2015). Recently it was detected that substantial amounts of starch can already be fermented in the SI (Awati et al., 2006). Fermentation of RS provides a large amount of SCFA readily utilized in the post-absorptive phase (Haenen et al., 2013). Lactic acid, acetate, propionate and butyrate are the main end products of microbial fermentation. In the pig, *Lactobacillus* dominate the stable microbial community causing lactic acid to be an important metabolic end product, which is rapidly used for butyrate production. Butyrate is the preferential substrate for gut epithelial cells and the relationship of SCFA production to health has been reviewed previously (Topping and Clifton, 2001). A plethora of data indicates that dietary RS increases SCFA concentrations in digesta and faeces (Bird et al., 2000; Bird et al., 2004) and increases net portal flux (Regmi et al., 2011b). Absorbed SCFA can be metabolized in many cell types providing prolonged energy. Occasionally, energy efficiency of RS is deemed to be similar to that of non-RS; thus, SCFA may provide energy more efficiently than mobilizing glucose from cell storage (Souza da Silva et al., 2014).

1.3.3 Microbial composition and potential energy harvest

Many bacteria ubiquitous to the pig hindgut possess enzymes capable of breaking down starch. However, limited data exists relating the gut microbial profile and hindgut nutrient availability and flow. High dietary amylose, or RS, has a bifidogenic effect in the proximal colon (Bird et al., 2007) and feces of pigs (Regmi et al., 2011). Furthermore, RS increased proximal colon and fecal *Lactobacill*us (Bird et al., 2007). Using human models, consumption of RS increased *Ruminococcus bromii* and *Eubacterium rectale* (Martínez et al., 2010). Similarly, retrograded starch (RS3) modulated microbiota profiles, increasing *Roseburia* spp. and *R. bromii* in pigs (Haenen et al., 2013; Umu et al., 2015). Of these studies, only Regmi et al., (2011) has correlated the abundance of Lactobacilli and bifidobacteria to increased post-ileal starch flow. How dietary-induced shifts in microbial profiles change energy harvest and host metabolism remains to be determined. Microbiota profiles enriched with Firmicutes have been recently associated with an increased capacity for energy harvest and weight gain (Ley et al., 2006; Turnbaugh et al., 2006). Specific phylotypes of bacteria (i.e., *Ruminococcus*) may also be instrumental in providing the host SCFA for energy recovery from RS (Ze et al., 2012; Umu et al., 2015). Thus, gut microbial profiles may shift towards specific phyla or species that will increase energy capture and the NE value of RS, a change that will be advantageous in maintaining pig growth.

Pig diets typically do not contain the purified RS that have been extensively studied. Cereal grains are the main source of starch for pigs and naturally have structural differences in their starch composition that will cause variation in host enzymatic hydrolysis potentially changing metabolic activity and composition of intestinal

microbiota (Reid and Hillman, 1999). Interestingly, a specialized barley cultivar high in amylose altered fermentation profiles vs. commercially-available barley evidenced by increased cecal and proximal colonic SCFA and decreased pH in pigs (Bird et al., 2004). However, this specialized high amylose barley did not cause major compositional changes in anaerobic, aerobic, coliform, or lactic acid bacteria indicating that differences in metabolism between purified and whole grain starch sources likely exist.

1.4 How to quantify digestion rate

1.4.1 Apparent total tract digestibility

Measurement of nutrient digestibility is the first step to determine the nutritive value of feedstuffs and provides a basis for diet formulation. Using total fecal collection or an indicator method with TiO_2, Cr_2O_3 or cobalt in feed, the ATTD of starch can be easily measured and has been broadly implemented (Lindahl, 1963). Characteristically, both RS and non-RS have near 100% disappearance rate by the distal colon or a 100% ATTD (Table 1.1). This makes it difficult to use ATTD of starch to accurately predict a feedstuff's energy value and subsequent animal performance. In accordance with the measured 100% ATTD of starch, NE prediction equations consider starch and sugars to be completely digestible explaining the use of total starch quantity in the equation (Noblet et al., 1994). Starch has an ATTD of $\geq 99\%$ in many cereal grains including corn, dehulled barley, dehulled oats, rice, rye, sorghum and wheat (Cervantes-Pahm et al., 2014). Even field pea with 48% RS and purified starch with 63% amylose has nearly 100% ATTD of starch (Sun et al., 2006a; Regmi et al., 2011a). Heat processing, known to affect SI starch digestion, does not affect ATTD of starch, where extruded vs. raw feedstuffs both have an ATTD of starch $\geq 96\%$ (Sun et al., 2006a).

Age of animals and resulting physiological status can also affect amount and kinetics of starch digestion. The period immediately following weaning is associated with changes in SI architecture and function due to villus atrophy and digestive enzyme depression (Lallès et al., 2004). During this post-weaning period, piglets have a decreased ability to digest and absorb nutrients including starch. However, physiological differences between grower and weaned piglets are not well illustrated when using the ATTD measures. As an example, feeding nursery pigs high amylose maize starch, hydrothermally treated high amylose maize starch or both, only minimally changed starch content in the distal colon, with ATTD of starch $\geq 83.2\%$ (Bird et al., 2007). A major limitation of using ATTD of starch is that SI digestion and hindgut fermentation are not separated. Even though all starch may have a 100% ATTD, differences in rate and site of starch disappearance between RS and non-RS can be detected in the SI and cecum of sows (Haenen et al., 2013). The energy value of starch digested in the SI vs. fermented in the hindgut will differ due to changes in energy lost through heat production. Thus, the use of ATTD of starch provides limited information to help formulate swine diets because it does cannot elucidate site, extent or kinetics of starch digestion.

1.4.2 Apparent ileal digestibility

The measure apparent ileal digestibility (AID) was first introduced by Low et al. (1980) and uses simple T-cannulas to elucidate small intestinal digestion. Extensively used in swine nutrition, T-cannulas can be inserted into the upper gastrointestinal tract (GIT) including the duodenum, jejunum, ileum and cecum and have been previously

reviewed (Knudsen et al., 2006). Surgical placement of T-cannulas into the distal ileum allows digesta to be sampled for determination of AID and nutrient flow. If both ileal digesta and faeces are collected one can distinguish between SI digestion and hindgut fermentation by the simple equation ATTD - AID. It is well established that intrinsic and extrinsic factors affect ileal digestibility of starch and changes in SI digestibility will have physiological and metabolic implications (Deng et al., 2010; Regmi et al., 2011b; Drew et al., 2012).

Consumption of high dietary amylose (63%) or RS (24%) vs. non-RS in purified starch sources decreased AID and increased post-ileal starch flow (Rideout et al., 2008; Regmi et al., 2011a). Even if ATTD of starch does not differ, the AID of RS is lower than non-RS (Rideout et al., 2008; Regmi et al., 2011a). The complex arrangement of starch within a cereal grain matrix can also modify the AID of starch and ranges in cereal grains from 85% in dehulled barley to 100% in wheat (Cervantes-Pahm et al., 2014). Other components, such as soluble fiber, within a cereal grain matrix aside from starch composition can affect starch digestibility. As an example, the AID of starch in corn (10.9% RS) was greater than that in dehulled barley (6.5% RS) (Cervantes-Pahm et al., 2014). Composition of feedstuffs can change physical characteristics of digesta (Lantle and Janssen, 2010), subsequently influencing nutrient digestibility. Barley is high in soluble fiber including β-glucans that can increase digesta viscosity and may decrease SI digestion. Extrinsic processing can also influence AID of starch, with extrusion increasing AID of starch from 33 to 98% post-extrusion (Sun et al., 2006a). The difference between incomplete SI starch digestion and 100% ATTD comes from hindgut fermentation. Fermentation begins at the distal ileum and continues through the hindgut.

Differences in rate of starch disappearance is noticeable well into the hindgut, where pigs fed high amylose corn vs. non-RS had increased cecal starch recovery up to 7.5 hours post-feeding (Topping et al., 1997). Using T-cannulas allows reliable and repeatable quantification of site, extent and kinetics of starch digestion and fermentation. One caution with surgical placement of cannulas is they may alter host physiology and consequently change post-cannula flow and recovery of nutrients.

An alternative method to cannulation to measure site, extent, and kinetics of digestion is to use the slaughter technique, which slaughters pigs 1 to 4 h post-prandially allowing for digesta sampling along the entire GIT (Bach Knudsen et al., 2006). The slaughter technique is commonly used on younger pigs, as cannulation is more difficult. Although this technique is criticized to introduce more variability and is a minute picture of digestion, it does not disrupt physiology of the GIT such as with cannulation surgery. Using the slaughter technique, intrinsic and extrinsic factors that affect AID of non-RS and RS in growing pigs are magnified in piglets. Starch recovery in the SI of piglets was measured to be 20-40 times greater when feeding retrograded RS vs. non-RS (Haenen et al., 2013). Similarly, feeding nursery pigs high amylose maize starch, hydrothermally treated high amylose maize starch or both vs. non-RS meaningfully reduced AID of starch to below 88 vs. 96% (Bird et al., 2007). Thus, pig age and physiological development play a role in starch and RS utilization and should be acknowledged when formulating weaned pig diets. Using cannulas, post-prandial slaughter or ATTD measures require the use of indigestible markers to calculate nutrient digestibility. However, indigestible markers can vary in their recovery rates based on feedstuff composition and the interactions with marker, thus making a suitable marker choice

difficult (Warner, 1981; Bach Knudsen et al., 2006). While cannulation and slaughter techniques allow quantification of site, extent and kinetics of starch digestibility the actual energetic value of starch remains undetermined.

1.4.3 Glycemic response

Consumption of RS changes the rate, site and kinetics of starch digestion, evidenced by lower AID of starch and increased post-ileal flow measures. This change in rate and site of digestion brought on by RS also directly impacts host physiology and metabolism (Deng et al., 2010; Regmi et al., 2011b; Drew et al., 2012; Giuberti et al., 2015). The rate of starch digestion is a main influencing factor in determining post-prandial glucose and insulin responses that were studied extensively in relation to metabolic diseases in humans (Mathers and Daly, 1998). The GI is a method used to define rate of starch digestion and its effect on resultant blood glucose levels and is stated to be a property of food (Jenkins et al., 1981; Jenkins et al., 1992). The GI can classify starchy foods and feedstuffs based on their rate of glucose absorption from the SI into low medium and high GI compared against a standard control (O'Dea et al., 1981). Evidently, RS with low AID of starch will limit the amount of glucose absorbed in the SI resulting in low GI and will consequently change the host's energy metabolism from glucose to SCFA (Topping, 1994).

Although the GI methodology has been extensively and effectively used for humans, use of GI methodology to predict rate of starch digestion in pigs is still novel and not widely adopted. Similar to humans, increasing dietary amylose from 3.1 to 64.1% decreased the GI in pigs from 85 to 30.9 respectively (Giuberti et al., 2012b). Similarly,

whole grains varied in their GI, with a slow rumen degradable barley having a GI of 49.4

and corn having a GI of 104.8 in pigs (Drew et al., 2012). Although not widely adopted,

the use of GI in pigs may be a low invasive alternative to cannulation to accurately

elucidate the rate of starch digestion and absorption in pigs. Rate of digestion has been

correlated with the GI of feedstuffs, with low GI barley having a slower digestion rate

than medium GI barley and high GI corn (Drew et al., 2012). The GI methodology is

continuously criticized for its validity, with questions raised about standardized methods

(Aziz, 2009), accuracy and precision of measurements (Xavier Pi-Sunyer, 2002), and

variations day to day, within, and between subjects (Devries, 2007). Although criticized,

a general consensus exists that GI methodology, if implemented and interpreted

correctly, is a valuable tool to predict SI digestibility (Wolever, 2013).

That starch with high amylose or RS content can reduce the *in vivo* glucose and

subsequent insulin response is generally accepted. However, some inconsistencies exist

between pigs (Noah et al., 1999; Regmi et al., 2011b) and humans (Higgins et al., 2004)

emphasizing the importance of adequate starch characterization, confounding feedstuff

components, and importantly the difference between glucose responses in peripheral vs.

portal circulation (Regmi et al., 2011b). To overcome problems associated with sampling

of peripheral vs. portal blood is to fit pigs with two indwelling catheters and a flow probe

around the portal vein (Rerat et al., 1984a,b). The catheterization model can repeatedly

quantify the kinetics of absorption of starch-derived nutrients and hormonal responses on

alive and conscious animals (Rerat et al., 1984a,b; Bach Knudsen et al., 2006). Using a

catheterization model with well-characterized purified starch sources has substantiated

previous evidence that starch composition alters glucose homeostasis, with consumption

of high vs. low amylose starch increasing net portal appearance of SCFA at the expense of glucose (Regmi et al., 2011b). Similarly using jugular catheters, high RS corn vs. low or non RS rice can also decrease post-prandial plasma glucose (Deng et al., 2010). A benefit of using catheters is that adequate blood can be sampled to measure nutrient and hormonal responses to RS and non-RS intake. Although use of peripheral blood to measure starch derived nutrients and hormonal responses has been criticized, combined use of portal vein and jugular catheters found that post-prandial insulin decreased in response to high dietary amylose and RS (Deng et al., 2010; Regmi et al., 2011a).

1.4.4 *In vitro* assays

Due to the intensive and invasive nature of *in vivo* techniques, *in vitro* methods estimating rate of starch digestion have been developed and extensively applied for humans and animals. The *in vitro* starch digestion techniques mimic *in vivo* digestion using timed enzymatic assays. The Englyst methodology, a classic *in vitro* fractionation method, measures glucose release from a feedstuff using glucose oxidase after digestion with amyloglucosidase and pancreatic invertase at 37°C (Englyst et al., 1992). This assay fractionates starch into three categories: rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) as the glucose released after 20, 100 and 120 min of digestion, respectively. The Englyst methodology is reliable and repeatable and has been extensively used to predict nutritional quality of starch in feedstuffs (Table 1.2). Although limitations do exist, the Englyst starch fraction RDS is positively correlated with *in vivo* glycemic responses for humans (Englyst et al., 1992; Goñi et al., 1997). Using the Englyst methodology, high RDS content in feedstuffs is indicative of a

high rate of SI starch digestion and increased post-prandial blood glucose level in comparison to fractions SDS and RS (Morand et al., 1992; Ells et al., 2005). Feedstuffs with greater SDS and RS content have delayed starch digestion, and can maintain lower post-prandial glucose responses (O'Dea et al., 1981, Muir and O'Dea et al., 1993). However, the Englyst starch fractions are criticized to not represent *in vivo* digestion kinetics and are unreliable predictors of RS and SDS when tested *in vivo* (Champ et al., 1998; Danjo et al., 2003; Rideout et al., 2008; van Kempen et al., 2010).

One criticism of the Englyst methodology is that the RS starch fraction does not truly resist SI digestion *in vivo*, with 60-70% of the *in vitro* RS fraction disappearing before the terminal ileum (Rideout et al., 2008). In both human and swine, RS digestion in the SI is thought to occur far beyond the 120 min time (Muir and O'Dea, 1993; van Kempen et al., 2010). Retention time through the SI of pigs is around 4 h (Wilfart et al., 2007), thus use of a longer digestion time of 240 min was proposed (Doti et al., 2014). Further extension of the Englyst methodology up to 8 h ensures 95% of the feedstuff is digested (van Kempen et al., 2010), with digestion beyond 8 h showing no change in *in vitro* SI RS digestibility (McCleary et al., 2002). A limit of using Englyst starch fractions is that they do not consider distal SI or colonic fermentation. In particular, the SI fermentation at the distal ileum is quite substantial (Bach Knudsen and Jørgensen, 2007). For the horse, another hindgut fermenter, non-structural carbohydrates (i.e., including starch) might be classified based on three fractions: hydrolysable, rapidly fermentable, and slowly fermentable, because energetic efficiency among these fractions differs (Hoffman et al., 2001). However, to date standardized methodology has not been adopted to measure these fractions. Criticisms aside, the RDS fraction of starch can be used with

moderate confidence and is a valuable tool to predict SI degradability and glucose

response of pigs to starchy feedstuffs.

1.4.5 *In vitro* kinetics model

The starch fractions RDS, SDS, and RS are often discussed independent of each

other; however, they are strongly related. Starch sources typically contain all three

fractions and cannot be easily be separated into the RDS, SDS, and RS components.

Before the Englyst starch fractions were developed, the moderately to high correlation

between rate of *in vitro* starch hydrolysis with peak glucose responses had been

established (O'Dea et al., 1981). *In vitro* kinetics models predict physiological responses

by measuring glucose release on a continuous curve vs. defining glucose availability-

based glucose released after arbitrary time points as in fractionation models. A kinetics

model using a modified Englyst assay corrected for gastric emptying can facilitate

accurate prediction of portal glucose appearance with an R^2 of 0.95 (van Kempen et al.,

2010). Using this *in vitro* kinetics model, it was shown that rapidly digested starch vs.

slowly digestible starch causes a 3-fold increase in glucose uptake *in vivo* (Regmi et al.,

2010). Metabolically, the increased peak glucose appearance of rapidly digestible starch

coincided with increased portal appearance of insulin (Regmi et al., 2010; Regmi et al.,

2011). Changes in peak glucose release *in vivo* are important to predict *in vitro* due to

their metabolic implications. Moderate insulin responses to glucose absorption are

required for glucose homeostasis and muscle growth; however, excess or prolonged

insulin release can cause increased lipid deposition and is a risk factor for metabolic

disorders. Thus, kinetics models displaying peak glucose release are beneficial to predict metabolic outcomes in monogastric livestock.

In pigs, *in vitro* hydrolysis indices have been strongly related to GI, with an R^2 of 0.95 (Giuberti et al., 2012b). Further modification and use of *in vitro* kinetics models allows calculation of a predicted glycemic index (pGI) that is associated with *in vivo* GI measurements: the pGI was 39.5 in corn and 80.0 in rice (Giuberti et al., 2012a). Supporting that heat treatment increases SI starch digestibility *in vivo*, the pGI increased from 39.5 to 100.8 in corn after heat processing (Sun et al., 2006a; Giuberti et al., 2012a). Thus, *in vitro* models displaying kinetics of glucose metabolism may have increased biological relevance in predicting host responses vs. fractionation methods. *In vitro* kinetics models can predict the gradual changes in glucose availability and peak glucose responses that are important physiological responses and parallel *in vivo* measurements.

1.4.6 Energy value

Using total collections to measure ATTD, cannulation and slaughter to measure AID and hindgut fermentation, peripheral blood collection or portal vein catheterization to measure nutrient and hormonal flux or *in vitro* analysis to predict SI digestion all provide insight on differences between RS and non-RS metabolism and the resulting nutritive value. However, the techniques reviewed come with their limitations, most importantly they cannot quantify the energy value of RS or non-RS. For accurate diet formulation and subsequent animal performance, quantification of the energy value of RS and non-RS is essential. Aside from the predictive NE equations published (NRC, 2012),

resources to calculate energetic value of RS or non-RS are limited. RS vs. non-RS differ in their site, extent and kinetics of digestion, which will ultimately affect energetic efficiency (van Kempen et al., 2010; Regmi et al., 2011b).

Using stoichiometric calculations in human nutrition, the DE value of RS was estimated to be only 50% of its GE value; however, this predicted DE value ignores hindgut fermentation and post absorptive metabolism (Livesey, 1990). In particular, conversion of SCFA from RS fermentation to retained energy might be only 70% of the enzymatically-digested end product glucose (Jørgensen et al., 1996; Noblet and van Milgen, 2004). Calorimetric methods are able to measure NE or energetic efficiency of feedstuffs by measuring energy loss through heat production directly or by inferring energy loss through gaseous exchange rates. Using calorimetry in humans and rats, the energetic value of RS was inconsistent and variable (Tagliabue et al., 1995; Ranhotra et al., 1996). Replacement of non-RS with RS increased energy excreted in faeces, urine and methane, causing the ME value of RS to be lower than non-RS (Schrama and Bakker, 1999). Interestingly, total heat production remained constant between RS and non-RS fed pigs; however, activity related heat production was reduced with RS consumption (Schrama and Bakker, 1999).

Using a novel calorimetry method based on differences in natural ^{13}C enrichment of feedstuffs, the energy value of starch and non-starch components was quantified precisely (Gerrits et al., 2012). In the novel method, the difference in NE between RS and non-RS was elucidated, with retrograded RS having only 83% of the NE value of non-RS (Gerrits et al., 2012b). Feeding fermentable carbohydrates has previously been associated with increased methane emissions (Jørgensen et al., 1996); however, methane

emissions did not differ when pigs were fed retrograded RS vs. non-RS (Gerrits et al., 2012b). Differences in methane emissions between types of RS indicate metabolism may change based on type of RS fed (i.e., RS1, RS2, RS3, etc.). Furthermore, the lack of change in methane emissions between retrograded RS and non-RS indicated that retrograded RS must have an alternative hydrogen sink such as breath excretion or reductive acetogenesis (Graeve et al., 1994; Gerrits et al., 2012a). Differences in RS metabolism based on starch type indicated a potential need to differentiate individually the differences in digestibility and resulting energy value between all types of RS vs. non-RS (Gerrits et al., 2012). When available calorimetric techniques are utilized to accurately quantify the energy value of RS and non-RS, more accurate diet formulation and better prediction of animal performance might be possible.

1.5 Growth performance and carcass composition

When evaluating feedstuffs, the nutritive value but also the feeding value or resulting animal performance must be determined. Rate and site of starch digestion may influence animal performance based on energetic efficiency of utilization. As discussed above, digestion vs. fermentation of starch can change efficiency of feedstuff utilization (Gerrits et al., 2012). In humans, consumption of SDS and RS will increase satiety, decreasing subsequent food intake and helping to maintain weight (Higgins, 2014). However in pigs, the evidence conflicts, despite repeatable substantiation in human studies (Higgins, 2014). Contradictory to studies in humans, feeding high RS field pea increased gain and feed to gain ratio over pigs fed RDS rice (Doti et al., 2014). Similarly, pigs fed SDS vs. RDS ad libitum also had increased feed intake and gain, likely due to decreased post-

prandial glucose release and subsequent satiety (van Kempen et al., 2007). Interestingly, a more recent study observed pigs fed RS ad libitum did not have decreased ADG or final BW despite lower DE intake, indicating energetic efficiency of RS may be similar to that of digestible starch (Souza da Silva et al., 2014). The increased energy retention found in pigs fed RS is proposed to be due to decreased activity-related heat production (Souza da Silva et al., 2014) that was shown previously (Schrama and Bakker, 1999). In contrast, pigs fed low GI cereal grains reduced N retention, indicating a potential to decrease lean growth (Drew et al., 2012). Furthermore, meal-feeding grower pigs high dietary amylose decreased gain and feed efficiency (Regmi et al., 2011b). The inconsistency in animal performance fed low GI, high RS or high amylose starch when meal feed vs. ad libitum indicate that physiological response may change based on feeding regime that should be considered carefully when designing research.

Carcass composition is an important factor in pork production, with a current market emphasis towards lean animals. In humans, consumption of RS may promote weight reduction through increased lipid oxidation, potentially resulting in leaner subjects (Higgins et al., 2004). In corroboration, feeding native RS and retrograded RS vs. non-RS in humans increased fat oxidation (Tagliabue et al., 1995; Achour et al., 1997). In pigs, consumption of fermentable carbohydrates increased lean deposition at the expense of fat deposition improving carcass grading in pigs (Szabó et al., 2007). The increased post-prandial blood glucose and insulin response to highly digestible starch may favor nutrient partitioning towards fat deposition (Roberts, 2000; Bolhuis et al., 2008). Thus, decreasing post-prandial insulin responses by feeding RS may be more effective for lean deposition (Doti et al., 2014) and may increase protein retention (Schrama and Bakker,

1999) while non-RS increased fat energy retention (Gerrits et al., 2012a). Although contradictory data exists, RS intake in pigs may increase growth performance and carcass composition of pigs, indicating that feedstuffs with high RS should be reconsidered for use in pig diets.

1.6 Conclusion

The North American swine industry gradually moves towards use of the NE system, which relies heavily on prediction equations to calculate the energy value of feedstuffs. Using the NE value of feedstuffs in feed formulation helps to accurately meet energy requirements of pigs, improving growth performance. The NE prediction equations published do not consider the actual energetic efficiency of starch, the main energy substrate in pig diets, and only considers the total quantity of starch. Many factors will change the rate, site and kinetics of starch digestion impacting host physiological and metabolic responses and subsequent energy value. Differences between RS and non-RS digestion will play an important role in pig body composition and subsequent growth performance. A multitude of reliable and repeatable *in vivo* and *in vitro* techniques are available for researchers to quantitatively determine the site, rate and kinetics of starch digestion; however, only indirect calorimetry can quantify the energy value of starch. The equations published in NRC (2012) will be used extensively in swine research, but determination of the NE value of RS and non-RS is recommended.

1.7 Knowledge gaps

Host physiological and metabolic responses differ according to the site and rate of starch digestion. Although purified starches with varying levels of RS have been studied extensively, only limited studies exist that associated the cereal grain matrix composition with starch digestibility, energy value and animal performance. Although RS may decrease AID digestibility of starch in both purified and whole grain sources, other compositional factors within cereal grains will also affect starch digestion including protein, lipid and fermentable fiber interactions. Cereal grains with high RS or fermentable fiber content have a lower energy value and thus are underutilized by the swine industry. There is a lack of understanding regarding how nutrient flow and substrate availability in the hindgut affects microbial composition and how a change in microbial composition subsequently alters host energy metabolism and growth potential. Dietary RS may in fact have nearly equal energetic efficiency as non-RS due to adequate provision of SCFA and decreased activity related energy loss (Gerrits et al., 2012b; Souza da Silva et al., 2014). Energetic efficiency aside, RS may also have a redeeming functional value and be physiological and metabolically beneficial to pigs in terms of gut health and their microbial composition. Thus, to characterize both the nutritive value and functional value of starch from purified and whole grain sources are important with an emphasis on metabolite and microbial composition.

1.8 Overall hypothesis and objectives

The hypotheses of this thesis were: a) Increasing amylose content of purified starch will change the site of nutrient digestion from the small intestine to the hindgut, shifting

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microbial and metabolite profiles of weaned pigs; b) Whole grains high in amylose and fermentable fiber will change the site of nutrient digestion from the small intestine to the hindgut, alter nutrient digestibility, shift microbial and metabolite profiles based on substrate availability in grower and nursery pigs; and c) Altering site and extent of starch digestion will cause changes in animal growth performance and energy efficiency of utilization (net energy) based on energy substrates utilized, glucose vs. SCFA.

The overall objective of the thesis was to elucidate the effect of chemical composition of purified starch and starch containing feedstuffs on the site and extent of nutrient digestibility, substrate flow through the GIT, its impact on microbial and metabolite profiles, and efficiency of energy utilization using a swine model. The specific objectives were:

1. To determine how increasing dietary amylose levels in weaned pigs will modify site and extent of starch digestion, feed efficiency, intestinal microbiota and metabolite profiles (Chapter 2).

2. To elucidate how differences in fermentable fiber composition of whole grain affect site and extent of nutrient digestion, standardized ileal digestibility (SID) of amino acids (AA), and whole body energy efficiency (NE) of grower pigs (Chapter 3).

3. To evaluate how whole grains differing in their fermentable fiber composition affect post-prandial nutrient flow through the GIT, substrate availability and microbial and metabolite profiles in the foregut and hindgut of grower pigs (Chapter 4).

4. To determine the interaction between fermentable vs. digestible whole grains and fermentable (aSBM) vs. digestible protein (SBM) on pig growth, site and extent of

nutrient digestion, nutrient flow through the GIT, microbial and metabolite profiles in the hindgut, and growth performance of weaned pigs (Chapter 5).

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Table 1.1 Starch apparent ileal digestibility (AID), apparent total tract digestibility (ATTD) and hindgut fermentation (ATTD-AID) according to starch source and pig age

Source	Pig Age	Processing	RS (%)	AID	ATTD	(ATTD-AID)	Reference
Barley	Grower	Raw	13	93	100	7	(Sun et al., 2006)
Barley	Grower	Extruded	2	99	100	1	(Sun et al., 2006)
Pea	Grower	Raw	48	78	99	21	(Sun et al., 2006)
Pea	Grower	Extruded	4	91	100	9	(Sun et al., 2006)
PSWB	Grower	Raw	47	33	96	63	(Sun et al., 2006)
PSWB	Grower	Extruded	2	98	100	2	(Sun et al., 2006)
Yellow dent corn	Grower	Raw	10.0	95.1	99.7	4.5	(Cervantes-Pahm et al., 2014)
Nutridense corn	Grower	Raw	10.9	98.5	99.9	1.5	(Cervantes-Pahm et al., 2014)
Dehulled barley	Grower	Raw	6.4	84.9	99.9	14.8	(Cervantes-Pahm et al., 2014)
Dehulled Oats	Grower	Raw	6.2	96.8	99.8	3.4	(Cervantes-Pahm et al., 2014)
Polished white rice	Grower	Raw	1.1	98.6	99.9	1.4	(Cervantes-Pahm et al., 2014)
Rye	Grower	Raw	11.7	92.3	99.6	7.4	(Cervantes-Pahm et al., 2014)
Sorghum	Grower	Raw	9.0	89.9	99.4	9.8	(Cervantes-Pahm et al., 2014)
Wheat	Grower	Raw	9.9	98.9	99.6	2.1	(Cervantes-Pahm et al., 2014)
HAMS	Nursery	Raw	-	87.8	93.5	5.7	(Bird et al., 2007)
HTHAMS	Nursery	Hydrothermal	-	69.7	83.2	15.5	(Bird et al., 2007)
HAMS+HTAMS	Nursery	Hydrothermal	-	73.5	84.7	11.2	(Bird et al., 2007)
Maize	Nursery	Cooked Flaked	-	97.3	-	-	(Vicente et al., 2009)
Rice	Nursery	Raw	1.83	98.3	-	-	(Vicente et al., 2009)
Rice	Nursery	Cooked	1.45	99.4	-	-	(Vicente et al., 2009)
Rice	Nursery	Cooked Flaked	1.95	98.7	-	-	(Vicente et al., 2009)
Barley	Nursery	Raw	-	97.2	-	-	(Medel et al., 2004)
Barley	Nursery	Cooked-flaked	-	98.3	-	-	(Medel et al., 2004)
Maize	Nursery	Raw	-	98.1	-	-	(Medel et al., 2004)
Maize	Nursery	Cooked-flaked	-	98.5	-	-	(Medel et al., 2004)

Table 1.2 *In vitro* starch fractions and GI of starchy feedstuffs

Feedstuff	Processing	Amylose	RDS (%)	SDS (%)	RS (%)	GI	Reference
Barley	Raw	-	9.8	45.6	8.1	-	(Sun et al., 2006a)
Barley	Extruded	-	60.8	9	13	-	(Sun et al., 2006a)
Peas	Raw	-	68	17.1	21.9	-	(Sun et al., 2006a)
Peas	Extruded	-	45.3	17	22	-	(Sun et al., 2006a)
PSWB	Raw	-	10.6	33.2	38.0	-	(Sun et al., 2006a)
PSWB	Extruded	-	81.9	9	19	-	(Sun et al., 2006a)
Starch	Raw	64.1	8.6	-	72.5	30.9	(Giuberti et al., 2012b)
Starch	Raw	26.8	11.1	-	53.5	43.3	(Giuberti et al., 2012b)
Starch	Raw	18.6	33.4	-	25.3	81.0	(Giuberti et al., 2012b)
Starch	Raw	3.1	51.4	-	20.1	85.0	(Giuberti et al., 2012b)
Starch	Raw	63.2	28.8	68.1	3.1	-	(van Kempen et al., 2010)
Starch	Raw	28.4	19.7	46.8	33.5	-	(van Kempen et al., 2010)
Starch	Raw	19.6	7.1	31.7	61.2	-	(van Kempen et al., 2010)
Starch	Raw	<0.5	3.6	11.4	85.0	-	(van Kempen et al., 2010)
Corn	Raw	-	-	-	-	104.8	(Drew et al., 2012)
Malting Barley	Raw	-	-	-	-	71.1	(Drew et al., 2012)
SRD Barley	Raw	-	-	-	-	49.4	(Drew et al., 2012)
Barley	Raw	-	23.2	53	23.8	-	(Doti et al., 2014)
Broken Rice	Raw	-	32.9	49.0	18.4	-	(Doti et al., 2014)
Maize	Raw	-	23.3	51.8	24.9	-	(Doti et al., 2014)
Peas	Raw	-	20.5	43.3	36.3	-	(Doti et al., 2014)
Maize	Raw	31.1[1]	14.7	36.7	19.1	39.5[2]	(Giuberti et al., 2012a)
Maize	Heat processed	-	60.3	7.4	11.3	100.8[2]	(Giuberti et al., 2012a)
Barley	Raw	28.9[1]	15.1	26.4	14.3	51.1[2]	(Giuberti et al., 2012a)
Barley	Heat Processed	-	48.4	10.3	3.2	96.9[2]	(Giuberti et al., 2012a)
Wheat	Raw	29.4[1]	18.1	37.8	5.3	65.8[2]	(Giuberti et al., 2012a)
Wheat	Heat Processed	-	43.8	23.6	70	77.1[2]	(Giuberti et al., 2012a)
Rice	Raw	23.3[1]	35.5	26.9	14.2	80.0[2]	(Giuberti et al., 2012a)

| Rice | Heat Processed | - | 70.4 | 59 | 89 | 106.9[2] | (Giuberti et al., 2012a) |

[1]Amylose content (g/100 g dry starch).
[2]pGI – predicted glycemic index.

a)
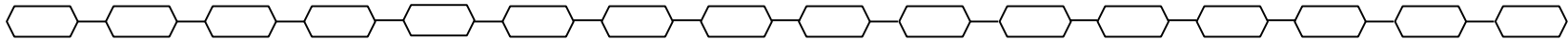
b)
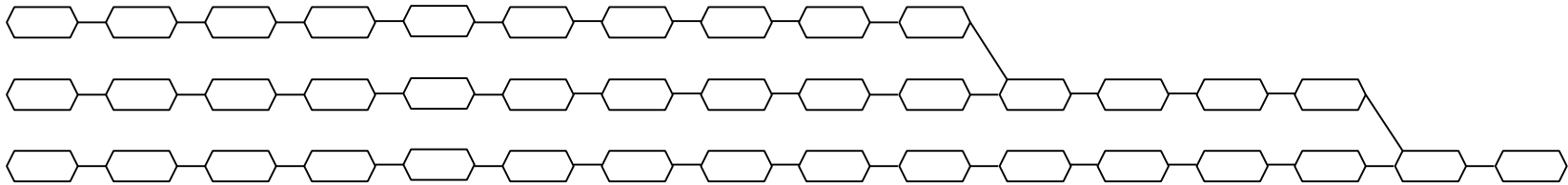
Figure 1.1 Schematic of a) amylose and b) amylopectin showing their linear and branched structure, respectively

Chapter 2. High amylose starch with low *in vitro* digestibility stimulates hindgut fermentation and has a bifidogenic effect in weaned pigs.

2.1 Introduction

Starch is an important source of energy in human and swine diets (1). Starch chemistry plays a role in the rate of starch degradation. Previously it had been accepted that the majority of starch was digested in the small intestine (1). However, a starch fraction called resistant starch (RS) has been defined as that portion of starch escaping small intestinal degradation and absorption, thus reaching the large intestine for use as a fermentative substrate (2). Increasing amylose, a type of RS, decreases digestibility by pancreatic α-amylase in the small intestine, thereby increasing digesta reaching the distal gut for microbial fermentation (3). Changes in substrate degradation by intestinal microbiota can induce changes in microbial and fermentation profiles. Previous studies have shown that increasing dietary amylose increases distal digesta mass, SCFA concentration, and commensal microbial populations, including bifidobacteria and lactobacilli (4–8). Increasing dietary amylose increases SCFAs (8) and consequently reduces gastrointestinal tract (GIT) pH (6, 7). Increases in SCFAs, particularly butyrate (9), promote growth and differentiation of enterocytes, and may increase intestinal integrity (5) and resistance against pathogenic microbes (10, 11). However, shifting pigs to starch fermenters vs. digesters may have an adverse effect on growth. Conversion of starch into SCFAs increases heat production and heat loss, thereby making SCFAs less energy efficient energy substrates than starch digestion products that are directly converted into ATP, which in concert may reduce growth of weaned pigs or humans.

The GIT is colonized with a diverse population of bacteria (12–14), some of which are mutualistic, defending against opportunistic pathogens and promoting physiologic and

immunologic functions (15–17). At weaning, piglets are exposed to stress from social, environmental, and nutritional changes. These stressors can lead to unstable gut microbial profiles, which are associated with decreased GIT integrity, malabsorption, postweaning diarrhea, and subsequent growth lags (18–21). Thus, dietary strategies to increase commensal microbiota and SCFA concentrations, potentially promoting gut health during weaning transition and recovery without compromising growth rate, are of interest.

Previously, studies have evaluated the effects of increasing dietary amylose on nutrient flow, microbiota, and fermentation metabolites in juvenile growing pigs (8, 22). However, no data have been published regarding how increasing inclusion of dietary amylose affects the GIT microbiota and environment and growth in pigs immediately after weaning. Manipulating the quantity of RS in diets consumed by weaned pigs may be a potential mechanism to promote GIT commensal microbiota and SCFA concentration (23); however, it may also limit growth through decreased efficiency of utilization of dietary energy. Our hypothesis was that increasing dietary inclusion of amylose would modify the extent to which diets were digested vs. fermented, feed efficiency, and the intestinal microbiota, and that these changes would be associated with changes in gut morphology, fermentation patterns, and pH in cecal and colonic digesta of pigs.

2.2 Methods

2.2.1 Pigs and diets

The animal use protocol was approved by the University of Alberta Animal Care and Use Committee for Livestock and followed the guidelines of the Canadian Council on Animal Care (24). A total of 32 crossbred barrows (Duroc sire × Large White/Landrace F_1, Genex Hybrid, Hypor; initial body weight, 7.1 ± 0.2 kg) were used for this experiment. Pigs were weaned at 21

d of age (d 0) and housed in individual metabolism pens (1.2 × 1.2 m) allowing freedom of movement, visual contact with other pigs, and free access to drinking water. Pigs were allocated to 1 of 4 test diets in a completely randomized block design. Diets were formulated to include 67% of 1 of the 4 purified starches (Table 2.1). The starch sources used in the 4 test diets were the same as previously described by Regmi et al. (22). The 4 starches used included Remyline AX-DR 0% amylose rice starch, Remy B7 20% amylose rice starch (both Remy Industries), Nastar 35.5% amylose pea starch (Cosucra Group), and Gelose 80% amylose corn starch (Penford Food Ingredients). Starches were previously characterized for amylose content, crystallinity, granule size, and *in vitro* digestibility (Table 2.2) (22). Starches were considered to be rapidly digestible (S1), moderately rapidly digestible (S2), moderately slowly digestible (S3), and slowly digestible (S4) based on maximal rate of *in vitro* starch digestion (S1, 1.06%/min; S2, 0.73%/min; S3, 0.38%/min; and S4, 0.22%/min) (25).

2.2.2 Sample collection

Pigs were acclimatized on a starter diet from day 0 to day 5, followed by 50% replacement of the starter diet by experimental diets on day 6. On day 7, 100% experimental diets were fed for a 3 wk period at 3 × the maintenance energy requirement [3 × 461 kJ digestible energy/kg BW$^{0.75}$] via bidaily feedings at 0800 and 1600 (26). Leftover and wasted feed were weighed back to determine average daily feed intake (ADFI). Pigs were weighed on days 0, 7, 14, and 21 to determine average daily gain (ADG). Subsequently, ADFI and ADG were used to calculate feed efficiency (gain/feed). On day 21, pigs were killed to collect duodenal, jejunal, ileal, cecal, and colonic digesta and tissue samples. Three hours post-feeding, pigs were sedated with 16 mg/kg body weight (BW) of ketamine HCl (Ketalar, Bioniche, Belleville, Ontario, Canada), 2.2 mg/kg BW of xylazine (Rompun, Bayer, Toronto, Ontario, Canada), and 8 mg/kg BW of azaperone

(Stresnil, Elanco, Guelph, Ontario, Canada) prior to killing with intracardial injection of 106

mg/kg BW of sodium pentobarbital (Euthanyl, Biomeda, Cambridge, Ontario, Canada) and

exsanguination. Pigs were placed in dorsal recumbency and a midline incision was made to

expose the internal body cavity and intestinal segments were clamped, dissected from the

mesentery, and removed. Individual segment lengths were measured. Cecal and colonic digesta

samples were snap-frozen in liquid N_2 and stored at -80°C for microbial and SCFA analyses with

the remaining digesta from all segments used to analyze pH. The distal 10 cm of the duodenum,

jejunum, ileum, and colon were preserved in zinc formalin for histology analysis.

2.2.3 Genomic DNA extraction and qPCR

Genomic DNA was extracted from cecal and colonic digesta of pigs with the use of a

QIAGEN QIAcube with a QIAamp DNA stool mini kit according to manufacturers instructions.

qPCR was performed on a StepOnePlus Real-Time PCR system (Applied Biosystems) with the

use of StepOne detection software (v2.1). Each reaction was run in duplicate in a volume of 20

µL in optical reaction plates sealed with optical adhesive film (Applied Biosystems). For SYBR

Green qPCR, reaction mixtures consisted of 10 µL QuantiFast SYBR Green Mastermix

(Qiagen), 1 umol (10 µmol/L) of primers (Table 2.3) (27–32), and 1 µL of template DNA of

cecal or colonic digesta samples. For Taqman qPCR, reaction mixtures consisted of 10 µL

Taqman Universal MasterMix (Applied Biosystems), 0.2 µL (10 µL umol/L) of primers, 0.08 µL

(10 µmol/L) probe, 0.04 µL ROX reference dye (Applied Biotsystems), and 1 µL of template

DNA of cecal or colonic digesta samples. Amplification was performed as previously described

(33). Standard curves were generated with the use of serial dilutions of the purified and

quantified PCR products generated by standard PCR with the use of specific primers and

genomic DNA from pig digesta.

2.2.4 Histology

Two transverse sections per pig of the duodenum, jejunum, and ileum were stained with the use of hematoxylin–eosin and analyzed with the use of a Nikon image analyzer. Ten well oriented villi and crypts were identified and villi height and crypt depth measured.

2.2.5 Chemical analysis

Samples of diets, freeze-dried ileal effluent, and feces were ground to pass through a 1.0 mm mesh screen (Lab Retsch Mill) before analyses of starch (Megazyme kit; Megazyme International), dry matter (DM), and titanium dioxide according to the methods of the AOAC (34). Concentration of SCFAs in cecal and colonic digesta was determined with the use of GC as previously described (35).

2.2.6 Calculations

Apparent ileal digestibility (AID) and apparent total tract digestibility (ATTD) of DM and starch were calculated for each pig based on the correction of TiO_2 content, using the equation:

$$AID \text{ or } ATTD \ (\%) = \{1-[(T_d/T_{f/j})/(N_d/N_{f/j})]\} \times 100$$

where T_d and $T_{f/j}$ are TiO_2 content of the diets and feces or ileal effluent, respectively, and N_d and $N_{f/j}$ are the nutrient contents in the diets and feces or ileal effluent, respectively. Hindgut fermentation of DM and starch in large intestine was calculated by subtraction of ATTD − AID. Ileal digesta flow of DM and starch was calculated for each pig using the equation:

$$\text{Ileal flow (mg/g DMI)} = [(100 - AID \% \text{ of nutrient}) \times \text{total daily intake of nutrient g} \times 10]/ DMI \ (g)$$

Feces output of DM and starch was calculated using the equation:

$$\text{Fecal output (mg/g DMI)} = [(100 - \text{ATTD \% of nutrient}) \times \text{total daily intake of nutrient}$$

$$\text{in g} \times 10]/ \text{DMI (g)}$$

2.2.7 Statistical analysis

Data were analyzed with the use of SAS version 9.1. The model included dietary treatment as a fixed effect and block as a random effect. Means were separated with the use of the PDIFF statement in the mixed model. Pig was considered the experimental unit. Significance of difference was set at P < 0.05 and a trend was set at P < 0.10. All variables were tested for normal distribution with the use of the Kolmogorov- Smirnoff test. Values are reported as means ± pooled SEMs. Regression analysis was used to estimate the linear and quadratic relation between increasing dietary amylose and dependent variables with the use of Proc Reg.

2.3 Results

2.3.1 Pig performance

Increasing dietary amylose linearly decreased ADG ($P < 0.05$) (Table 2.4) and quadratically reduced ADFI ($P < 0.05$). Increasing dietary amylose quadratically decreased AID of starch and DM and ATTD of DM ($P < 0.05$) and quadratically increased ileal digesta flow of starch and DM and fecal DM output ($P < 0.05$). Increasing dietary amylose linearly reduced ATTD of starch ($P < 0.05$) and linearly increased fecal starch output ($P = 0.05$). Increasing dietary amylose quadratically increased hindgut fermentation of DM and starch ($P < 0.05$).

2.3.2 Microbial profile

Gene copy numbers (wet basis) of *Enterobacteriaceae* and *Bacteroides* groups in cecal

digesta did not differ in pigs consuming the 4 diets (Table 2.5). Gene copy numbers (wet basis) of *Enterobacteriaceae* in colonic digesta also did not differ in pigs consuming the 4 diets. Pigs that consumed the S1 and S4 diets had increased gene copy numbers (wet basis) of the *Bacteroides* group in colonic digesta ($P < 0.05$) vs. pigs that consumed the S2 and S3 diets. Pigs that consumed the S3 diet had decreased gene copy numbers (wet basis) of the *Lactobacillus* group in cecal and colonic digesta ($P < 0.05$) vs. pigs that consumed the remaining 3 diets. Increasing dietary amylose quadratically increased *Bifidobacterium* spp. gene copy numbers (wet basis) in cecal and colonic digesta ($P < 0.05$). Increasing dietary amylose quadratically decreased *Clostridium* cluster XIVa and IV gene copy numbers (wet basis) in cecal and colonic digesta ($P < 0.05$).

2.3.3 Fermentation characteristics

Increasing dietary amylose quadratically increased cecal and colonic propionate and valerate concentration (wet basis) ($P < 0.01$) (Table 2.6). Butyrate and BCFA (isobutyrate and isovalerate acid) concentrations (wet basis) in cecal and colonic digesta did not differ among pigs consuming the 4 diets. Acetate concentration (wet basis) in cecal digesta did not differ among pigs that consumed the 4 diets. However, increasing dietary amylose linearly increased acetate concentration (wet basis) in colonic digesta ($P < 0.05$). Pigs that consumed the S4 diet had decreased cecal digesta caproate concentrations (wet basis) ($P < 0.001$) compared with pigs consuming the remaining 3 diets, with pigs that consumed the S3 diet having increased caproate concentrations ($P < 0.001$) vs. those consuming the S1 and S2 diets. Colonic digesta caproate concentration (wet basis) did not differ among pigs consuming the 4 diets ($P > 0.10$). Increasing dietary amylose quadratically decreased ileal, cecal, and colonic digesta pH ($P < 0.05$) (Table 2.7). Duodenal digesta pH did not differ among pigs consuming the 4 diets. Stomach pH was

increased in pigs that consumed the S2 and S4 diets ($P < 0.05$) vs. pigs that consumed the S1 and S3 diets.

2.3.4 Gastrointestinal architecture

Duodenum, jejunum, and ileum villi height and crypt depth did not differ among pigs consuming the 4 diets ($P > 0.10$) (Table 2.8). In the duodenum, increasing dietary amylose quadratically reduced villus height (P < 0.05) and linearly increased crypt depth ($P < 0.05$). Pigs that consumed the S3 and S4 diets had an increased duodenum length ($P < 0.05$) in comparison with those that consumed the S1 and S2 diets (29.8 and 29.1 cm vs. 25.4 and 25.9 cm $\pm$ 2.12 pooled SEM, respectively). The length of the jejunum, ileum, and colon did not differ among pigs that consumed the 4 diets.

2.4 Discussion

The present study evaluated effects of increasing dietary inclusion of amylose at constant dietary starch levels on growth and gut starch flow, microbes, metabolites, and histology in pigs immediately after weaning. To determine the effects of amylose alone, purified starch sources were used to avoid the confounding effects of intrinsic starch-associated compounds, including fat, fiber, and protein. Pigs are used as a model for humans to understand the effects of diet on host responses (36).

2.4.1 Growth

Meal-feeding slowly digestible starch decreased growth and feed efficiency in rats (37–39) and pigs (6). In the present study, increasing dietary amylose reduced ADG and ADFI, similar to previous research in which the same starch sources were fed to heavier pigs (8). In the present

study, decreased growth in pigs consuming increasing dietary amylose was associated with decreased ileal starch digestion that increased ileal starch flow into the distal gut and starch fermentation. Hindgut fermentation of fiber or starch, followed by absorption and metabolism of SCFAs, provides maximally 50–60% of metabolic energy vs. when starch and sugars are digested and absorbed in the small intestine (40). This loss of energetic efficiency may account for the reduced feed efficiency observed in the present study. RS has a satiating effect presumably because of increased postprandial peripheral SCFAs and subsequent increases in satiety hormones (41). Because maintaining growth rate is a key goal when feeding both weaned pigs and infants, regardless of potential benefits incurred when feeding 63% amylose, reduced growth is not an ideal outcome. In contrast to the present study with meal-fed pigs, ADFI was previously the greatest for weaned pigs with free access to diet containing moderately slowly digestible starch vs. rapidly digestible starch (42). Although the meal-feeding used in the present study modeled after human eating habits may be an excellent predictor for human infants, it is less ideal for predicting feed intake and growth of pigs that produce pork. Pigs consuming the S3 diet in the present study had lower digestibility of DM and starch than did juvenile pigs consuming the same starch in a previous study (8). The lower digestibility of DM and starch in the S3 diet in present study can be attributed to younger age, GIT developmental state, and site of ileal digesta sampling. Terminal ileal cannulas used in the previous study (8) allowed sampling to occur at the distal ileum, whereas we sampled throughout the entire ileum to obtain sufficient sample for analyses, decreasing digestibility.

2.4.2 Microbial profile

Microbial composition is dependent on substrate availability and microbial substrate preference (43). Dietary intervention often aims to increase host commensal microbiota, such as

48

Bifidobacterium and *Lactobacillus,* because they positively influence immune function and GIT development while inhibiting pathogenic microbes (44, 45). With increasing dietary amylose, *Bifidobacterium* spp. increased and *Clostridia* clusters IV and XIVa decreased in cecal and colonic digesta. Reduced *Clostridia* cluster XIVa was previously reported when pigs consumed RS, which may be attributed to lack of competitiveness of the cluster, because members may depend on substrate availability and pH (46, 47). Previously, juvenile pigs consuming the S4 diet had increased *Bifidobacterium* spp. without other population changes detected in feces or ileal digesta (8). Consumption of diets containing 45% high amylose starch and hydrothermally treated high amylose starch increased *Bifidobacterium* spp. and the *Lactobacillus* group in pigs (6). However, we only observed a decrease in the Lactobacillus group with pigs consuming the S3 diet in cecal and colonic digesta. In the present study, pigs consuming the S4 diet had an increased *Bacteroides–Prevotella–Porphyromonas* cluster in colonic digesta, indicating that amylose acts as a fiber-like fermentative substrate, because these bacteria are adapted to utilize fiber (44). Both *Clostridia* clusters I and XI were below detection limit in the present study. These clusters include both commensal and pathogenic species, the latter being potentially harmful to the host (48). *Enterobacteriaceae*, a large family of bacteria that includes well-known pathogens such as Salmonella and *Escherichia coli,* remained unchanged, indicating that weaned pigs in the present study were likely not challenged by pathogenic bacteria and had adequate time to recover from weaning stress. The increases observed in commensal *Bifidobacterium* spp. and ileal starch flow while increasing dietary amylose were linear, indicating that microbial profiles depend on amount and rate of starch entering the hindgut for fermentation.

2.4.3 Fermentation characteristics and pH

Digesta SCFA concentration is also related to the amount of substrate available for

microbial fermentation. In the present study, increasing dietary amylose increased hindgut fermentation, as evidenced by increased total SCFA concentrations in cecal and colonic digesta. Previously, consumption of 42.5% and 30% dietary RS increased total SCFA concentration in pigs (6, 47). Moreover, increasing dietary amylose increased concentrations of propionate and valerate in cecal and colonic digesta and of acetate in colonic digesta. This shift in metabolites matches the increased abundance of *Bifidobacterium* and the *Bacteroides–Prevotella–Porphyrmonas* cluster, because their major metabolites include acetate and propionate. Similarly, gut propionate concentration increased when high amounts of amylose (49) and RS (47) were fed. Butyrate concentration changes were not observed in cecal or colonic digesta among pigs consuming the 4 diets. Conversely, RS consumption increased in butyrate concentrations in the proximal intestine (6) and colon (47). Butyrate is the preferred metabolic fuel of colonocytes regulating proliferation and differentiation (50). Lower butyrate concentrations in cecal and colonic digesta may not reflect production, because butyrate uptake and metabolism may have increased with increased production (8, 11). Members of *Clostridium* cluster Cl. XIVa, known butyrate producers, decreased as dietary amylose content increased in the present study, further explaining the lack of change in cecal and colonic digesta butyrate concentrations (51). Increased luminal SCFA concentration lowers pH, which may inhibit pathogen colonization (52, 53). In the present study, higher intraluminal SCFA concentration was associated with lowered pH in digesta. Similarly, consumption of high amylose starch lowered digesta and fecal pH of pigs (6) and rats (37).

2.4.4 Gastrointestinal histology

Microbial fermentation products, SCFAs, promote mucosal epithelium proliferation by increasing GIT length, weight (11), and villus height. Increasing GIT surface area benefits host

metabolism by increasing absorptive capacity. In the present study, duodenum length increased when pigs consumed the S3 and S4 diets, indicating amylose may have a trophic effect. Similarly, RS intake and colon length were related in 49-d-old pigs (6) that had an altered gross morphology. The microscopic structure of the mid-colonic wall did not change as pigs consumed increasing dietary amylose in the present study, similar to previous research in which microstructure changes were not observed in pigs consuming high amounts of dietary RS (6). In the present study, piglets were 47 d of age when killed, substantially past the initial acute deterioration immediately post-weaning, indicating that their GITs have had considerable time to recover from weaning stress and return to full integrity (19).

2.5 Conclusion

Dietary inclusion of amylose acts as a fermentative substrate and changes young pigs from starch digesters into starch fermenters, evidenced by an increase in total SCFA concentration in cecal and colonic digesta. The increased fermentative activity was associated with increased *Bifidobacterium* spp. and acidification of the GIT, each of which may benefit gut health in young pigs; however, these effects came at the cost of reduced growth. This indicates that dietary amylose starch has a lower net energy than high amylopectin starch per weight unit of starch in pigs with restricted access to feed. Although starch with 63% amylose may not be useful to promote optimal growth rates in young pigs, this starch may be useful to humans interested in maintaining body weight through increased satiety and reduced energetic efficiency. Increasing dietary amylose was linearly related to growth and gut health indexes; thus, an ideal dietary amylose inclusion may exist to optimize growth and gut health in the newly weaned pig and human infant.

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Table 2.1 Ingredient and formulated nutrient composition of the test diets

Ingredient	Inclusion rate
	g/kg
Starch source[1]	670
Casein[2]	180
Fish meal[3]	75
Cellulose[4]	30
Canola oil	10
Limestone	10
Mono-dicalcium phosphate	7
Vitamin premix[5]	5
Mineral premix[6]	5
NaCl	3
TiO_2	3
K_2CO_3	2
Formulated nutrient composition (as is)	*g/kg*
Crude protein	215
Lys	16.9
Met	5.94
Thr	9.83
Trp	2.72
NE, MJ/kg	9.58

[1] S1, Remyline AX-DR rice; S2, Remy B7 rice; S3, Nastar pea; or S4, Gelose 80 corn.

[2] Calcium caseinate, American Casein Company, Burlington, NJ.

[3] Menhaden meal, Omega Protein, Hammond LA.

[4] Solka-floc, International Fiber Corp, North Tonawanda, NY.

[5] Provided per kg diet: 100 mg $ZnSO_4$; 80 mg $FeSO_4$; 50 mg $CuSO_4$; 25 mg $MnSO_4$; 0.5 mg $Ca(IO_3)_2$; 0.1 mg Na_2SeO_3.

[6] Provided per kg diet: 2.5 mg retinol; 20.6 μg cholecalciferol; 2.7 μg d,l-α-tocopherol; 35 mg niacin; 15 mg D-pantothenic acid; 5 mg riboflavin; 4 mg menadione; 2 mg folic acid; 1 mg thiamine; 0.2 mg D-biotin; and 0.025 mg vitamin B_{12}.

Table 2.2 Characteristics of the 4 starch sources used in experimental diets fed to weaned pigs for 21 d[1]

| Characteristics (DM basis) | Starches | | | |
	S1	S2	S3	S4
Source	Rice	Rice	Field pea	Corn
Digestion rate[2], $g/(kg \times min)$	10.6	7.3	3.8	2.2
Starch, g/kg	963	958	979	943
Amylose content[3], %	0	20	28	63
Crystallinity[4], %	40	36	30	24
Granule width[5], μm	1.8-3.8	2.4-5.6	5.0-13.5	3.4-10.9
Granule length[5], μm	2.4-5.7	2.9-8.9	5.4-34.0	3.5-16.6

[1] Reproduced with permission from Regmi et al., 2011 (22).
[2] Maximum *in vitro* rate of digestion, determined using a 10 hour enzymatic digestion.
[3] Determined using a Megazyme amylose/amylopectin assay kit.
[4] Determined using X-ray diffraction.
[5] Determined by scanning electron-microscopy.

Table 2.3 Oligonucleotide primers used to profile cecal and colonic digesta from pigs consuming over 21 d 4 diets containing starch sources differing in amylose content and thus in rates of *in vitro* digestion

Targeted bacterial group	Primer size (bp)	Orientation[a]	Primer sequence (5'-3')	Annealing temp (°C)	Reference
Lactobacillus group[1]	341	F	AGCAGTAGGGAATCTTCCA	62	(27)
		R	CACCGCTACACATGGAG		
Bifidobacterium spp.	84	F	GCGTGCTTAACACATGCAAGTC	60	(28)
		R	CACCCGTTTCCAGGAGCTATT		
		P	TCACGCATTACTCACCCGTTCGCC		
Bacteroides group[2]	140	F	GGTGTCGGCTTAAGTGCCAT	60	(29)
		R	CGGAYGTAAGGGCCGTGC		
Clostridium cl. I	120	F	ATGCAAGTCGAGCGAKG	60	(29)
		R	TATGCGGTATTAATCTYCCTTT		
Clostridium cl. IV	239	F	GCACAAGCAGTGGAGT	60	(30)
		R	CTTCCTCCGTTTTGTCAA		
Clostridium cl. XIVa	438-41	F	AAATGACGGTACCTGACTAA	60	(30)
		R	CTTTGAGTTTCATTCTTGCGAA		
Clostridium cl. XI	104	F	ACGCTACTTGAGGAGGA	60	(31)
		R	GAGCCGTAGCCTTTCACT		
Enterobacteriaceae	195	F	CATTGACGTTACCCGCAGAAGAAGC	63	(32)
		R	CTCTACGAGACTCAAGTTGC		

[1] *Lactobacillus* spp., *Pediococcus* spp., *Weissella* spp., and *Leuconostosc* spp.

[2] *Bacteroides-Prevotella-Porphyrmonas*; F = forward primer; R = reverse primer; P = probe.

Table 2.4 Growth over 21 d and nutrient digestibility of weaned pigs consuming 4 diets containing starch sources differing in amylose content and thus rates of *in vitro* digestion[1]

Characteristic	Starch diets				Pooled SEM	ANOVA *P*-value	Linear		Quadratic	
	S1	S2	S3	S4			R^2	*P*-value	R^2	*P*-value
ADG, *g/d*	318[a]	330[a]	311[a]	272[b]	15.1	0.014	0.13	0.040	0.14	0.11
ADFI, *g/d*	469[a]	472[a]	465[a]	447[b]	7.2	0.041	0.19	0.012	0.21	0.030
Efficiency, *gain/feed*	0.68[a]	0.70[a]	0.67[a]	0.61[b]	0.028	0.024	0.09	0.080	0.10	0.21
AID, %										
DM	93.6[a]	91.4[a]	88.2[b]	83.3[c]	0.90	<0.001	0.60	<0.001	0.62	<0.001
Starch	97.5[a]	96.8[a]	88.9[b]	78.4[c]	1.78	<0.001	0.55	<0.001	0.55	<0.001
Ileal digesta										
DM flow, *mg/g DMI*	63.6[b]	87.0[b]	127.8[a]	156.8[a]	10.17	<0.001	0.60	<0.001	0.61	<0.001
Starch flow, *mg/g DMI*	15.5[b]	22.1[b]	85.4[a]	118.4[a]	14.39	<0.001	0.54	<0.001	0.54	<0.001
ATTD, %										
DM	97.5[a]	97.2[a]	97.6[a]	95.4[b]	0.09	<0.001	0.54	<0.001	0.61	<0.001
Starch	100.0	100.0	100.0	99.9	0.02	0.11	0.13	0.041	0.17	0.069
Feces										
DM output, *mg/g DMI*	25.4[b]	27.9[b]	26.8[b]	43.4[a]	2.20	<0.001	0.54	<0.001	0.61	<0.001
Starch output, *mg/g DMI*	0.1	0.1	0.1	0.5	0.14	0.13	0.13	0.046	0.17	0.073
ATTD-AID, %										
DM	3.8[a]	5.8[a]	9.3[b]	12.1[c]	0.81	<0.001	0.53	<0.001	0.57	<0.001
Starch	2.4[a]	3.5[a]	11.2[b]	21.4[c]	1.64	<0.001	0.55	<0.001	0.55	<0.001

[1] Values are means and pooled SEM, *n* = 8. Means in a row with superscripts without a common letter differ, *P* < 0.05. ADG, average daily gain; ADFI, average daily feed intake; AID, apparent ileal digestibility; ATTD, apparent total tract digestibility; DM, dry matter; S1, S2, S3, and S4, respectively, refer to rapidly digestible, moderately rapid digestible, moderately slow digestible, and slowly digestible starch based on maximum rate of *in vitro* starch digestion.

Table 2.5 Bacterial groups in cecal and colon digesta of weaned pigs consuming over 21 d 4 diets containing starch sources differing in amylose content and thus rates of *in vitro* digestion[1]

Characteristic	Starch diets				Pooled SEM	ANOVA *P*-value	Linear R^2	Linear *P*-value	Quadratic R^2	Quadratic *P*-value
	S1	S2	S3	S4						
	log₁₀ 16S rRNA gene copies/g wet weight									
Bacterial groups in cecal digesta										
Lactobacillus group	8.22[a]	8.49[a]	7.50[b]	8.20[a]	0.205	0.016	0.02	0.46	0.03	0.60
Enterobacteriaceae	8.69	9.16	9.07	9.37	0.297	0.33	0.07	0.15	0.09	0.24
Bacteroides group	10.6	10.4	10.4	10.7	0.12	0.41	0.02	0.42	0.14	0.11
Clostridium cl XIVa	9.82[a]	9.39[ab]	9.06[b]	8.19[c]	0.263	<0.001	0.43	<0.001	0.44	<0.001
Clostridium cl IV	9.35[a]	8.96[b]	8.87[b]	8.44[c]	0.146	0.001	0.26	0.003	0.31	0.004
Bifidobacterium spp.	5.39[bc]	5.09[c]	5.99[b]	6.91[a]	0.317	0.003	0.26	<0.003	0.27	0.012
Clostridium cl I	BDL	BDL	BDL	BDL	-	-	-	-	-	-
Clostridium cl XI	BDL	BDL	BDL	BDL	-	-	-	-	-	-
Bacterial groups in colon digesta										
Lactobacillus group	7.88[a]	7.81[a]	6.72[b]	7.93[a]	0.309	0.025	0.01	0.61	0.05	0.49
Enterobacteriaceae	8.35	8.92	8.84	8.87	0.333	0.31	0.04	0.25	0.12	0.18
Bacteroides group	10.7[ab]	10.5[b]	10.5[b]	10.9[a]	0.13	0.052	0.06	0.182	0.19	0.050
Clostridium cl XIVa	9.96[a]	9.58[a]	9.28[a]	8.24[b]	0.297	<0.001	0.41	<0.001	0.41	<0.001
Clostridium cl IV	9.62[a]	9.05[a]	9.05[a]	8.45[b]	0.213	0.005	0.34	<0.001	0.35	0.002
Bifidobacterium spp.	5.47[bc]	4.89[c]	6.01[b]	7.50[a]	0.338	<0.001	0.33	<0.001	0.38	<0.001
Clostridium cl I	BDL[2]	BDL	BDL	BDL	-	-	-	-	-	-
Clostridium cl XI	BDL	BDL	BDL	BDL	-	-	-	-	-	-

[1] Values are means and pooled SEM, *n* = 8. Means in a row with superscripts without a common letter differ, *P* < 0.05. BDL, below detection level of 2 log₁₀ 16S rRNA gene copies/g (wet weight); S1, S2, S3, and S4, respectively, refer to rapidly digestible, moderately rapid digestible, moderately slow digestible, and slowly digestible starch based on maximum rate of *in vitro* starch digestion.

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Table 2.6. SCFA concentration in cecal and colon digesta of weaned pigs consuming over 21 d 4 diets containing starch sources differing in amylose content and thus rates of *in vitro* digestion[1]

Characteristic	Starch diets				Pooled SEM	ANOVA *P*-value	Linear		Quadratic	
	S1	S2	S3	S4			R^2	*P*-value	R^2	*P*-value
μmol/g wet matter										
Cecal digesta										
Acetate	42.6	38.6	37.1	42.4	3.23	0.56	0.00	0.79	0.11	0.20
Propionate	11.9[b]	13.0[b]	12.3[b]	47.8[a]	1.49	<0.001	0.66	<0.001	0.78	<0.001
Butyrate	5.5	4.9	5.0	4.0	0.72	0.52	0.08	0.12	0.08	0.30
Isobutyrate	0.2	0.2	0.1	0.0	0.13	0.62	0.06	0.17	0.06	0.40
Valerate	0.8[b]	0.9[b]	1.0[b]	2.9[a]	0.35	0.001	0.34	<0.001	0.40	<0.001
Isovalerate	0.0	0.1	0.1	0.0	0.10	0.52	0.00	0.84	0.07	0.36
Caproate	0.5[ab]	0.1[b]	0.6[a]	0.0	0.15	0.018	0.05	0.22	0.05	0.48
Total	61.5[b]	57.7[b]	56.3[b]	97.1[a]	4.65	<0.001	0.40	<0.001	0.56	<0.001
Colonic digesta										
Acetate	29.5[b]	32.0[b]	38.4[b]	44.6[a]	3.59	0.032	0.13	0.045	0.14	0.107
Propionate	5.7[b]	8.5[b]	12.2[b]	31.5[a]	3.01	<0.001	0.42	<0.001	0.42	<0.001
Butyrate	3.8	4.7	4.2	5.2	0.84	0.49	0.04	0.30	0.04	0.55
Isobutyrate	0.3	0.0	3.9	6.8	4.09	0.49	0.05	0.21	0.06	0.43
Valerate	0	0.3[b]	0.8[b]	3.7[a]	0.49	<0.001	0.33	<0.001	0.33	0.003
Isovalerate	0.2	0.0	0.0	0.3	0.15	0.49	0.02	0.42	0.07	0.34
Caproate	0.3	1.1	4.4	7.2	1.58	0.28	0.08	0.10	0.03	0.23
Total	40.0[c]	47.0[bc]	64.1[b]	92.9[a]	7.32	<0.001	0.38	<0.001	0.38	<0.001

[1] Values are means and pooled SEM, n = 8. Means in a row with superscripts without a common letter differ, P < 0.05. SCFA, short chain fatty acid; S1, S2, S3, and S4, respectively, refer to rapidly digestible, moderately rapid digestible, moderately slow digestible, and slowly digestible starch based on maximum rate of *in vitro* starch digestion.

Table 2.7. Gastro-intestinal pH of weaned pigs consuming over 21 d 4 diets containing starch sources differing in amylose content and rates of *in vitro* digestion

Segment	Starch diets				Pooled SEM	ANOVA P-value	Linear		Quadratic	
	S1	S2	S3	S4			R^2	*P*-value	R^2	*P*-value
Stomach	4.3[b]	4.7[a]	4.4[b]	4.8[a]	0.11	0.005	0.11	0.058	0.13	0.14
Duodenum	5.4	6.2	5.5	5.5	0.28	0.23	0.07	0.14	0.09	0.24
Ileum	8.1[a]	7.8[a]	6.7[b]	6.7[b]	0.22	<0.001	0.36	<0.001	0.41	<0.001
Cecum	6.7[a]	6.8[a]	6.6[a]	5.2[b]	0.24	<0.001	0.43	<0.001	0.50	<0.001
Colon	6.6[a]	6.3[a]	6.6[a]	5.3[b]	0.16	<0.001	0.49	<0.001	0.58	<0.001

[1] Values are means and pooled SEM, n = 8. Means in a row with superscripts without a common letter differ, P < 0.05. S1, S2, S3, and S4, respectively, refer to rapidly digestible, moderately rapid digestible, moderately slow digestible, and slowly digestible starch based on maximum rate of *in vitro* starch digestion.

Table 2.8. Histology of weaned pigs consuming over 21 d 4 diets containing starch sources differing in amylose content and thus rates of *in vitro* digestion[1]

Characteristic	Starch diets				Pooled SEM	ANOVA P-value	Linear R^2	Linear P-value	Quadratic R^2	Quadratic P-value
	S1	S2	S3	S4						
Duodenum										
Villus height, *μm*	761[a]	653[b]	663[b]	704[a]	30.9	0.090	0.01	0.62	0.20	0.036
Crypt depth, *μm*	104	115	112	115	5.9	0.52	0.13	0.045	0.13	0.14
Jejunum										
Villus height, *μm*	637	612	607	595	50.2	0.93	0.01	0.54	0.01	0.86
Crypt depth, *μm*	109	129	106	100	9.4	0.18	0.07	0.15	0.05	0.46
Ileum										
Villus height, *μm*	502	523	583	516	30.8	0.29	0.01	0.58	0.01	0.89
Crypt depth, *μm*	99	108	103	113	8.5	0.28	0.04	0.30	0.01	0.86

[1] Values are means and pooled SEM, $n = 8$. Means in a row with superscripts without a common letter differ, $P < 0.05$. S1, S2, S3, and S4, respectively, refer to rapidly digestible, moderately rapid digestible, moderately slow digestible, and slowly digestible starch based on maximum rate of *in vitro* starch digestion.

Chapter 3. Effect of cereal grain fiber composition on site of nutrient digestion, standardized ileal digestibility of amino acids, and whole body energy utilization in grower pigs

3.1 Introduction

Swine diets are conventionally formulated with cereal grains such as wheat and barley to provide starch as the main energy source. Barley can vary greatly in nutrient composition dependent upon cultivar and agronomic conditions (Fairbairn et al., 1999). Within barley, high levels of amylose and non-starch polysaccharides (**NSP**) including β-glucans can reduce apparent ileal digestibility (**AID**) of nutrients and energy (Montagne et al., 2003; Högberg and Lindberg, 2004; Metzler-Zebeli and Zebeli, 2013). Subsequently, more undigested nutrients flow into the hindgut becoming available for microbial fermentation to provide a major supply of energy to the pig. However, hindgut carbohydrate fermentation causes energy loss through heat production and reduced utilization of fermentation products (Noblet and Le Goff, 2001) compared to small intestinal starch digestion.

Previous studies have examined how individual components including amylose (Bird et al., 2007; Regmi et al., 2011), β-glucan (Hahn et al., 2006; Metzler-Zebeli and Zebeli 2013) or fermentable fiber (Hooda et al., 2011) can affect nutrient digestibility. However, little information is available about the impact of naturally-occurring ratios of amylose, β-glucan, and total dietary fiber (**TDF**) within the cereal grain matrix on the site and extent of energy and nutrient digestibility, N metabolism, and NE value. The swine industry is moving toward adoption of the NE system to formulate diets, therefore

quantification of the NE value of energy substrates becomes more important. More precise methods are now necessary to predict NE values, since traditional methods may overestimate NE value of feedstuffs (NRC, 2012).

We hypothesized that barley cultivars containing high fermentable fiber, namely β-glucans, change the site of nutrient digestion from the foregut to hindgut in grower pigs, and thus reduce AA digestibility and whole body energy utilization. The objective of the present study was to characterize the energetic value of barley varying in chemical composition compared to wheat.

3.2 Materials and methods

Experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock. Pigs were handled in accordance with the guidelines described by the Canadian Council on Animal Care (CCAC, 2009). The animal study was conducted at the Swine Research and Technology Centre (Edmonton, AB, Canada).

3.2.1 Animals, cereal grains, and diets

Seven crossbred F_1 barrows (initial BW of 27.7 ± 1.6 kg, final BW 116.5 ± 5.1 kg; Large White × Landrace) were surgically fitted with a T-cannula at the distal ileum, with cannula dimensions and surgical procedure previously described (Sauer and Ozimek, 1986). Pre and postoperative care has also been described (Li et al., 1993). After surgery, barrows recovered for 7 d with a gradual increase in feed allowance before being switched to the first assigned experimental diet. Pigs were housed individually in

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metabolism pens (1.2 m wide, 1.2 m long and 0.9 m high) to allow freedom of movement in a thermo-controlled room ($22 \pm 2°C$). Pens were composed of plastic-coated expanded metal floors, polyvinyl chloride walls with Plexiglass windows (0.3×0.3 m). A stainless-steel feeder and cup drinker were attached to the front of the pen.

As whole grain, 3 hull-less-barley cultivars; high-fermentable high β-glucan (HFB, CDC Fibar), high-fermentable high amylose (HFA, CDC Hilose), moderate-fermentable (MFB, CDC McGwire) and 1 low-fermentable hard red spring wheat (LFW, CDC Utmost) were obtained from the Crop Development Centre (Saskatoon, SK, Canada) and 1 low-fermentable hulled-barley, (LFB, Xena) was obtained from Viterra (Wetaskiwin, AB, Canada). Cereal grains were ground with a hammer mill using a 2-mm screen to prepare diets (Table 3.1). Five diets were formulated to include 1 of 5 cereal grains with a 'protein component' made up of fishmeal, soy protein concentrate, crystalline AA, canola oil, vitamin and mineral premix, and Cr_2O_3 to meet or exceed nutrient requirements for growing pigs (NRC, 2012). A basal diet was formulated to include the same 'protein component' as in the 5 cereal grain diets with an identical ratio of protein sources and canola oil. The basal diet had an identical ratio of cornstarch, dextrose, and cellulose as the N-free diet to allow for calculation of digestibility of the 'protein component' according to the difference method. Using digestibility of the 'protein component', digestibility of the cereal grain was then calculated according to the difference method (Bureau et al., 1999).

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3.2.2 Experimental procedure

The study was conducted as a 6 (period) × 7 (diet) Youden square to obtain 6 observations per diet. Each period lasted 14 d and daily feed allowance was set at 2.4 × 197 kcal DE/kg $BW^{0.60}$ via 2 daily feedings at 0800 and 1600h (NRC, 2012). Diets were fed as a dry mash, and pigs had free access to water throughout the experiment. Each period consisted of a minimum 5 d acclimation to the experimental diets, followed by a 9 d collection that included: respiration measurements via indirect calorimetry, and collection of urine, feces, and ileal digesta. Pigs rotated through indirect calorimetry chambers for energy metabolism measurements and metabolism pens for urine and fecal collections, followed by ileal digesta collection.

Feces and urine were collected simultaneously for 48 h and kept frozen until homogenization for freeze-drying. Urine was filtered through cheesecloth and collected twice daily following feeding from jugs containing 10 mL HCl to minimize volatilization of urine N as ammonia. Aliquots of urine, 10% of total weight, were obtained, pooled for the collection, and stored at -20°C. Feces were collected continuously in plastic bags fitted around the anus that were replaced minimally twice per day, pooled for each pig and period were stored at -20°C as previously described (Van Kleef et al., 1994).

Gas exchange was determined over 24 h period immediately following either the 5 d adaptation or feces and urine collection using open circuit indirect calorimetry. Air was drawn through boxes at rates of ~250 L/min. Airflow was measured after passing drawn air through a cold water condenser and commercial air meters (Model 1023, Canadian Meter Corp., Cambridge, ON, Canada). A sample of air was drawn with a small air pump (Model 0531, Gast Mfg. Corp., Benton Harbour, MI) and delivered to a

fuel cell type O_2 analyzer, a non-dispersive near infrared CO_2 analyzer and a CH_4 analyzer (Qubit Systems, Kingston, ON, Canada). Airflow to the analyzers was regulated to 0.5 L/min by ball type flow meters (Scienceware Size 2, Fisher Scientific, Mississauga, ON, Canada). The analog output (mV) of the analyzers was converted to digital data by an analog digital converter (Qubit Systems) and recorded in 1 min intervals. Gas analyzers were calibrated for zero and gain readings with pure N_2 (zero) or calibration gas (1.5% CO_2, 21% O_2, 100 ppm CH_4, balance N_2) each study day. Gas measurements at steady state were recorded pre and post observation period. During respiration measurements, pigs were moved to respiration chambers 2 h before the evening meal to allow for adaptation to the chamber for 12 h. On the following morning expired air was continuously analyzed for O_2, CO_2, and CH_4 content over 24 h (fed state). Water was freely available in the chambers. Room temperature was kept at 23-24°C and personnel movement around the chambers was limited to avoid disturbances.

Ileal digesta was collected continuously for 2 d, for 8 h daily from 0800 to 1600 h as previously described (Seneviratne et al., 2010). Collected digesta was pooled for each pig and period and stored frozen at -20°C. Prior to chemical analysis, feces and digesta were thawed, homogenized, subsampled, and freeze-dried.

3.2.3 Chemical analysis

Diets, cereal grains, and lyophilized digesta and feces were ground in centrifugal mill (Retch model ZMI; Brinkman Instruments, Rexdale, ON, Canada) through a 1 mm screen. Diets, cereal grains, digesta, and feces were analyzed for GE using an adiabatic bomb calorimeter (model 5003; Ika-Werke GMBH & Co. KG, Staufen, Germany), DM

by drying at 135°C in an airflow type oven for 2 h (method 934.01), CP by oxidation (N × 6.25 GP-428 N determinator; Leco Corporation, St Joseph, MI) (method 984.13A-D), ether extract using diethyl ether as solvent (EE; method 920.39A), ash (method 942.05), ADF (method 973.18A-D), NDF (Van Soest et al., 1991), Ca (method 968.08), P (method 946.06), AA by ion-exchange chromatography (method 982.30E), and chemically-available Lys (method 97.5.44) content using standard methods (AOAC, 2006). Diets, digesta, and feces were analyzed for Cr_2O_3 by spectrophotometry (model 80-2097-62, KBUltraspec III; Pharmacia, Cambridge, UK) at 440 nm after ashing at 450°C overnight (Fenton and Fenton, 1979), β-glucan (Mixed linked beta-glucan Kit; Megazyme International), and starch (Megazyme Total Starch kit; Megazyme International). Urinary N was analyzed using Shimadzu total organic carbon analyzer (Model TOC-V CHS/CSN, Shimadzu Corp. Kyoto, Japan).

3.2.4 Calculations

The index method was used to calculate digestibility of nutrients in diets. Apparent ileal digestibility (AID) and apparent total tract digestibility (ATTD) of nutrients in diets were calculated using the following equation (Adeola, 2001):

ATTD or AID, % = 100 – [100 × (concentration of Cr_2O_3 in diet × concentration of nutrient in feces or digesta/ concentration of Cr_2O_3 in feces or digesta × concentration of nutrient in diet)].

The basal endogenous loss of an AA or CP (g/kg DMI) was calculated by the equation for the N-free diet, Eq. [3] of (Stein et al., 2007)

I_{end} = AA or CP in digesta × (Cr_2O_3 in diet/ Cr_2O_3 in digesta).

Basal endogenous loss estimates from pigs fed the N-free diet were used for the correction of AID coefficients to derive standardized ileal digestibility (SID) values for each indispensable AA using the following Eq. [7] of (Stein et al., 2007)

$$SID = AID + (I_{end}/AA_{diet} \text{ in diet})$$

Digestibility of cereal grains was calculated according to the difference method applied twice. The 'protein component' (23% CP and contained fishmeal, soy protein concentrate, crystalline AA, canola oil, vitamin and mineral premix, and Cr_2O_3) digestibility was first determined according to the difference method from Eq. [2](Bureau et al., 1999):

$$D_{protein\ component} = D_{basal\ diet} + [(D_{basal\ diet} - D_{N\text{-}free\ diet}) \times (0.62 \times N_{N\text{-}free\ diet} /0.38 \times N_{protein\ component})],$$

where $D_{protein\ component}$ = % digestibility of the protein component, $D_{basal\ diet}$ = % digestibility of the basal diet, $D_{N\text{-}free}$ diet = % digestibility of the N-free diet, 0.62 means 62% of the N-free diet in the basal diet, 0.38 means 38% of protein component in the basal diet $N_{N\text{-}free}$ diet = % nutrient (or Kcal/kg GE) of the N-free diet (DM basis), and $N_{protein\ component}$ = % nutrient (or Kcal/kg GE) of protein component (DM basis).

Digestibility of the cereal grains was then calculated according to the difference method applied again (Bureau et al., 1999):

$$D_{cereal\ grain} = D_{diet} + [(D_{diet} - D_{protein\ component}) \times (0.20 \times N_{protein\ component}/0.80 \times N_{cereal\ grain})],$$

where $D_{cereal\ grain}$ = % digestibility of the cereal grain, D_{diet} = % digestibility of the cereal grain diet, $D_{protein\ component}$ = % digestibility of the 'protein component', 0.2 means 20% of the protein component in the cereal grain diets, 0.8 means 80% of cereal grains in the cereal grain diets, $N_{protein\ component}$ = % nutrient (or Kcal/ kg GE) of the protein component

(DM basis), and $N_{cereal\ grain}$ = % nutrient (or Kcal/kg GE) of cereal grain (DM basis). The NE values were calculated using Eq. [1-7] and Eq. [1-8] adapted from (Noblet et al., 1994) in (NRC, 2012).

The NE calculations were adapted from equations previously described (Moehn et al., 2013). Briefly, heat production (HP) was calculated based on gas exchange (indirect calorimetry) according to (Brouwer, 1965) as:

$$HP\ (MJ/d) = 16.18 \times O_2 + 5.02 \times CO_2 - 5.99 \times U_N - 2.17 \times CH_4,$$

where O_2, CO_2 and CH_4 are the gas exchanges in L/day, and U_N is the urinary nitrogen excretion in g/d, which was converted into Kcal/d. The respiratory quotient, RQ, was calculated as CO_2 production divided by O_2 consumption. Protein retention was calculated using the N balance technique and reported as N retention. The ME intake was calculated as DE intake minus CH_4 energy (39.56 kJ/L) and urinary energy, which was calculated using C:N ratio of 0.9:1, as kJ/g = 0.333 × % C + 0.093 × % N as previously described (Moehn et al., 2013). Energy retention was calculated as ME intake minus heat production. Dietary NE was calculated as retained energy plus maintenance energy expenditure, estimated at 197 Kcal/kg $BW^{0.60}$ (NRC, 2012). To determine the NE value of cereal grains, the difference method was applied as previously described.

3.2.5 Statistical analysis

Data were analyzed using PROC MIXED of SAS (version 9.3; SAS inst. Inc., Cary, NC). Confirmation of normality was completed using PROC UNIVARIATE. Diet was a fixed effect with pig and period random effects. Diet fed in the previous period was used as covariate to test for carryover effects. Least squares means were reported.

Multiple comparisons between least squares means were achieved using the PDIFF statement with TUKEY adjustment. Significance was declared at $P < 0.05$.

3.3 Results

Pigs remained healthy and consumed their daily feed allowance regardless of diet offered during the study.

3.3.1 Chemical Composition of Cereal Grains

Cereal grains were regarded as low, medium, and high fermentability based on their β-glucan content (Table 3.2). Chemically-available Lys was similar among cereal grains. The CP content was greater for HFB and LFW than the other 3 cereal grains. Diet CP content ranged from 22.5% for LFB to 27.1% for HFB (Table 3.3). Diet NDF content was greatest in HFB (28.6%) and diet ADF content was greatest in LFB (4.81%). Cellulose addition in the basal and N-free diet increased their ADF content to 6.36 and 4.62%, respectively. Starch content in diets ranged from 41.4% (HFA) to 50.6% (LFW). Diet β-glucan content ranged from 0.88% (LFW) to 8.54% in HFB.

3.3.2 Nutrient digestibility of cereal grains

The ATTD–AID (hindgut fermentation) of GE, DM, starch, and β-glucan was greatest ($P < 0.05$; Table 3.4) in HFB and HFA vs. MFB, LFB, and LFW. The ATTD–AID of CP was greatest ($P < 0.05$ in HFB and HFA vs. MFB, LFB, and LFW. The ATTD–AID of CP was greatest ($P < 0.05$) in HFB and HFA vs. LFB and LFW. The ATTD–AID of GE, DM, and starch were linearly related to the β-glucan content of the

cereal grains and aligned with their assigned low, medium, and high fermentability descriptors (Figure 3.1). The AID of GE and CP and ATTD of CP was greatest ($P <$ 0.05) in LFW vs. HFB and HFA. However, the ATTD of GE was less ($P < 0.05$) in LFB than the other cereal grains. The AID of DM and β-glucan was greatest ($P < 0.05$) in LFW, LFB, and MFB vs. HFB and HFA. The ATTD of DM was greater ($P < 0.05$) for MFB than LFB. The AID of starch was greater ($P < 0.05$) for LFW and LFB vs. HFB and HFA; however, the ATTD of starch did not differ ($P > 0.05$) among cereal grains.

3.3.2 SID of cereal grains

Similar to ATTD and AID of CP, the SID of CP, total AA, Ile, Leu, Met, Phe, Glu, and Tyr was greatest ($P < 0.05$) in LFW, but lowest ($P < 0.05$) in HFB and HFA (Table 3.5). The SID of Arg, His, Thr, Trp, Val, Ala, Asp, Cys, Gly, Pro, and Ser were greatest ($P < 0.05$) in LFW, LFB, and MFB but lowest ($P < 0.05$) in HFB and HFA. The SID of Lys was greater ($P < 0.05$) in LFB and MFB than HFB.

3.3.3 Energy metabolism

The FHP, calculated from prediction equations, did not differ ($P > 0.05$; Table 3.6) among diets. Consumption of O_2 and resulting heat production (HP) was greatest ($P < 0.05$) for LFW and HFA diets vs. HFB and LFB diets. However, CO_2 production did not differ ($P > 0.05$) among diets. The CH_4 production was greatest ($P < 0.05$) for HFB and HFA diets vs. the LFW diet. The CH_4 production was linearly related ($R^2 = 0.20$; $P <$ 0.024) to hindgut fermentation of starch (Figure 3.2). The RQ was greatest ($P < 0.05$) for the HFB diet vs. the LFW diet. Also, retained energy was greatest ($P < 0.05$) for the HFB

diet vs. HFA and LFB diet. The DE and ME content were least ($P < 0.05$) for hulled

barley, LFB, among cereal grains, causing the ratio of ME to DE to be greatest ($P < 0.05$)

for LFW and least ($P < 0.05$) for HFA. Only small differences in NE content were

observed, with NE of HFB greater ($P < 0.05$) than HFA. Since HFA had the least NE, the

ratio of NE to ME was least ($P < 0.05$) for HFA and greatest ($P < 0.05$) for HFB.

3.4 Discussion

3.4.1 Nutrient digestibility of cereal grains

Starch is the main carbohydrate and energy source in cereal grains. Cereal grains

contain starch within a complex matrix of protein, fiber, and lipids that interact to affect

solubility, viscosity, and fermentability, and therefore subsequently alter nutrient

digestibility and energy value (Dikeman and Fahey, 2006). From the present study,

changing site of nutrient and energy digestion from the small intestine to the hindgut was

specific to cereal grain cultivar and fermentable fiber content, specifically β-glucan

content. The lower AID of DE, DM, starch, CP, and β-glucans in HFB and HFA vs.

MFB, LFB, and LFW can be attributed to increased fermentable fiber content, mainly β-

glucans that replaced starch. Previously, barley with low starch and high NDF content

had decreased AID of DM, CP (McCann et al., 2006), and GE (Pettersson and Lindberg,

1997). Despite lower AID of starch and β-glucan in HFB and HFA, a compensating

increase in hindgut fermentation of these nutrients resulted in all cereal grains in the

present study having equivalent ATTD of starch and β-glucans. The high amylose

content in HFA likely caused increased hindgut fermentation of starch, as amylose is

highly fermentable within the hindgut but resists small intestinal digestion (Regmi et al.,

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2011). In the present study, cereal grain β-glucan content was linearly related to hindgut fermentation of nutrients, explaining the increased hindgut fermentation of nutrients and energy in HFA and HFB. Barley has a high water-binding capacity (Cervantes-Pahm et al., 2014), thought to be important for increasing viscosity (Souffrant, 2001), and increasing hindgut nutrient digestion (Pettersson and Lindberg, 1997).

Dietary inclusion of barley fiber can increase endogenous N losses (Leterme et al., 2000; Souffrant, 2001), explaining the lower AID and ATTD of CP in HFB, HFA, MFB, and LFB vs. LFW. Hindgut fermentation of nutrients can increase microbial N utilization, stimulating N flux from microbial protein into the lumen (Canh et al., 1998), explaining lower ATTD of CP in all barley diets vs. LFW. The low fermentability of barley hulls explains the least ATTD of GE and DM, thus leading to a lower DE value, for hulled barley (LFB) vs. all other cereal grains. These findings are similar to previous studies indicating that inclusion of barley hulls (Bell et al., 1983) or use of hulled barley vs. hull-less barley decrease a feeds energy value (Pettersson and Lindberg, 1997).

3.4.2 SID of cereal grains

Feed specific endogenous losses were included into total endogenous outflow, potentially underestimating the SID of N and AA. Feed specific losses of Thr, Gly, Pro, and Asp pose the most concern due to their abundance in endogenous protein (Jondreville et al., 2001). The SID of Pro in MFB, LFB, and LFW exceeded 100%, indicating an overestimation of endogenous losses. This phenomenon has also been previously observed (Stein et al., 2001). Increased SID of CP, total AA, and most individual AA in LFW, LFB, and MFB vs. HFB and HFA can be ascribed to the greater fermentable fiber

content in HFB and HFA specifically the β-glucan content. Fermentable fiber content, such as β-glucans, can range from 5-11% in barley cultivars (Skendi, 2003) and can resist digestion depending on their arrangement within the cereal grain matrix (Holtekjølen, 2014). High levels of fermentable fiber in barley cultivars HFA and HFB may have caused the reduced SID of AA, since increasing levels of dietary fiber reduces SID of AA (Ma et al., 2008). The negative effect of fiber on SID of AA can be attributed to the physical link between fiber and CP in the cereal matrix, decreasing both enzymatic access to the substrate and its hydrolysis (Bach Knudsen, 2001). Furthermore, fiber can decrease nutrient digestibility by increasing endogenous AA secretions and mucus production, increasing the digesta passage rate through the SI (Stanogias and Pearcet, 1985). Although LFB has high TDF content, it likely is contained predominantly in the hull and not the kernel matrix, thus decreasing the amount of interference of fiber on SID of AA as was observed with the hull-less barley cultivars, HFB and HFA.

In the present study, SID of AA from LFW were comparable to those previously reported for wheat in growing pigs (Stein et al., 2001). The SID of AA from MFB and LFB were comparable, but on average slightly less than those previously reported for barley in growing pigs (Stein et al., 2001; Brestenský et al., 2013). Previously, variations in SID of AA in barley among studies has been attributed to differences in endogenous losses, and less so to variations in nutrient content (Fan et al., 1995). The current findings emphasize the importance of considering fiber content of cereal grains when assessing and describing variations in SID of AA.

3.4.3 Energy metabolism

The mean NE value of hulled barley, hull-less barley, and wheat is 2.34, 2.46, and 2.47 Mcal/kg respectively (NRC, 2012). Predictive equations employed in the present study found comparable values, however indirect calorimetry measured NE was less than predicted values. Differences in predicted vs. measured NE may be attributed to an increased energy loss from the higher protein content of LFW and fermentable fiber content of the barley cultivars fed in the present study. Increasing CP intake has previously shown to decrease both retained energy (RE) and NE through reduced efficiency of energy utilization (Moehn et al., 2013). In the present study, increased N intake of pigs consuming LFW increased heat production and decreased NE value in comparison to the NE value reported for wheat in the NRC tables. Similarly, overfeeding protein increases energy expenditure (Bray et al., 2015) through increased heat production (Halton and Hu, 2004). Although HFB had comparable CP to LFW, its lower protein AID and SID values likely explain the decreased energy lost through protein oxidation and was supported by the lower heat production measured. Lower O_2 consumption and RQ (1.18) in pigs consuming HFB may indicate a surplus of carbohydrate that cause lipid deposition and change in substrate oxidation from that of protein (McClave et al., 2003).

High levels of fermentable fiber in the cereal grains in the present study can be a further cause for lower measured vs. predicted NE values. An increased CH_4 production in pigs consuming HFB and HFA vs. LFB and LFW supports the theory of hindgut fermentation. In agreement with the present study, CH_4 emissions increased linearly with increasing fermentable carbohydrate intake (Jorgensen, 2007). Amylose content in barley

starch is highly variable, ranging from 3-46% (Björck, 1990) and can be encapsulated within a rigid protein matrix or cell wall, thus inhibiting small intestine digestion. Previously, resistant starch has only 83% of the NE value of digested starch (Gerrits et al., 2012). Interestingly, the current study found that heat production was greater in pigs fed high amylose barley, HFA, vs. HFB, which is in disagreement with literature that found resistant starch intake did not change heat production (Gerrits et al., 2012) or in fact lowered thermogenesis (Tagliabue et al., 1995). In the present study, activity related heat production was not measured and may have been a contributing factor to differences observed in heat production and overall NE value of feedstuffs, since activity related heat production changed between digestible and resistant starch intake (Gerrits et al., 2012). Pigs consuming HFA, MFB, and LFB had a RQ near 1 which may indicate oxidation of carbohydrates, in agreement with previous work finding pigs fed resistant starch to have RQ of 0.96 – 1.00 (Gerrits et al., 2012). Variation in the NE value of cereal grains may also be attributed to changes in FHP due to increasing feeding levels throughout the present study, as feeding level is known to alter FHP (de Lange et al., 2006; Labussière et al., 2010). Differences between predicted and measured NE value of feedstuffs (Table 3.7) warrants further investigation regarding the accuracy of predictive equations when feedstuffs are high in fermentable fiber or digestible protein.

3.5 Conclusion

Cereal grains high in fermentable fiber, namely β-glucans, moved the site of energy digestion in pigs from the foregut to the hindgut. Furthermore, the high β-glucans content in specific hull-less barley cultivars is an influential factor that decreases SID of AA. The

increased levels of fermentable fiber and digestible protein also had an effect on the measured NE value of the cereal grains. Consequently, predictive equations calculating the NE of feedstuffs high in fermentable fiber and resistant starch (amylose) may grossly underestimate energy losses due to increased thermogenesis. Although barley and wheat may be suitable feedstuffs to provide the main energy and partial source of protein in swine diets, careful consideration of fermentable fiber content is advised. Current findings suggest specific varieties of cereal grains high in fermentable fiber may be limiting in SID AA, thus using them in diet formulations may require judicious titration of protein feedstuffs to ensure maintenance of growth.

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Table 3.1 Ingredient composition of experimental diets (as-fed basis)

Ingredient, %	Test diet	Reference diets Basal	N-free
Cereal grain[1]	80.00	–	–
Dextrose	–	25.46	38.00
Corn starch	–	25.46	38.00
Soy protein concentrate[2]	7.22	15.64	–
Fishmeal	7.22	15.64	–
Solka-Floc[3]	–	11.07	16.50
Canola oil	1.70	2.22	3.30
Monodicalcium phosphate	0.80	1.00	0.93
Limestone	0.60	0.40	0.90
Vitamin premix[4]	0.50	0.50	0.50
Mineral premix[5]	0.50	0.50	0.50
Cr_2O_3	0.50	0.50	0.50
Salt	0.40	0.40	0.30
L-Lys•HCl	0.35	0.76	–
L-Thr	0.13	0.28	-
DL-Met	0.05	0.11	-
L-Trp	0.03	0.06	-
KCO_3, 56%K	-	-	0.40
MgO, 58%Mg	-	-	0.10
Choline chloride	-	-	0.07

[1]Five diets were formulated with 1 of 5 cereal grains. High-fermentable high β-glucan hull-less barley (HFB, CDC-Fibar), high-fermentable high amylose hull-less barley (HFA, CDC-Hilose), moderate-fermentable hull-less barley (MFB, CDC-McGwire), and low-fermentable wheat (LFW, CDC-Utmost) were obtained from Crop Development Centre (Saskatoon SK, Canada). Low-fermentable hulled barley (LFB, Xena) was obtained from Viterra (Wetaskawin AB, Canada).

[2]Hamlet Protein 300, Hamlet Protein Inc. Findlay, OH.

[3]International Fiber Corp., North Tonawanda, NY.

[4]Provided the following per kilogram of diet: vitamin A, 8,250 IU; vitamin D_3, 825 IU; vitamin E, 40 IU; 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; and vitamin B_{12}, 0.025 mg.

[5]Provided the following per kilogram of 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 $Ca(IO_3)_2$; and Se, 0.1 mg as Na_2SeO_3.

Table 3.2 Analyzed composition of cereal grains[1] (DM basis)

Item, %	Hull-less barley			Hulled barley	Wheat
	HFB	HFA	MFB	LFB	LFW
DM	90.3	90.3	89.7	89.5	89.3
GE, Mcal/kg	4.56	4.57	4.50	4.43	4.49
CP (N × 6.25)	19.6	16.7	16.1	14.2	19.7
Ether extract	0.98	1.77	0.17	0.00	0.00
TDF	22.4	18.4	14.6	17.0	14.1
ADF	5.49	6.17	6.17	9.40	6.44
NDF	26.3	23.8	21.2	24.7	25.9
Starch	52.9	54.2	58.5	62.7	61.0
Amylose	0.19	18.0	11.4	11.2	12.3
β-glucan	10.33	7.42	4.92	4.41	0.73
Ash	2.43	2.24	1.64	2.42	2.02
Chemically-available Lys	0.60	0.61	0.51	0.47	0.47
Indispensable AA					
Arg	0.78	0.70	0.63	0.53	0.72
His	0.38	0.32	0.30	0.26	0.39
Ile	0.61	0.50	0.48	0.45	0.61
Leu	1.17	1.00	0.95	0.86	1.19
Lys	0.60	0.61	0.51	0.47	0.47
Met	0.28	0.24	0.22	0.20	0.28
Phe	0.95	0.71	0.72	0.65	0.85
Thr	0.54	0.50	0.43	0.39	0.49
Trp	0.16	0.14	0.14	0.12	0.23
Val	0.79	0.70	0.65	0.60	0.71

[1]HFB = high-fermentable high β-glucan hull-less barley; HFA = high-fermentable high amylose hull-less barley; MFB = moderate-fermentable hull-less barley; LFB = low-fermentable hulled barley; LFW = low-fermentable wheat.

Table 3.3 Analyzed nutrient content and GE value of cereal grain based diets[1] (DM basis)

Item, %	Hull-less barley			Hulled barley	Wheat	Reference diets	
	HFB	HFA	MFB	LFB	LFW	Basal	N-free
DM	90.9	91.0	90.6	90.3	90.1	94.5	95.2
GE, Mcal/kg	4.55	4.52	4.49	4.46	4.49	4.29	4.07
CP (N × 6.25)	27.1	23.1	23.3	22.5	26.1	23.0	2.17
Ether extract	4.06	4.59	4.39	3.84	3.62	3.73	1.26
ADF	2.51	2.51	2.20	4.81	3.16	6.36	4.62
NDF	28.6	15.9	13.1	15.4	13.2	15.1	7.4
Starch	41.7	41.4	47.4	46.6	50.6	23.6	33.8
β-glucan	8.54	5.79	4.13	3.65	0.88	0.24	0.36
Ash	6.25	6.14	6.34	6.69	6.34	6.98	3.15
Chemically-available Lys[2]	1.23	1.28	1.20	1.28	1.25	1.77	0.02
Indispensable AA							
Arg	1.13	1.07	1.03	1.04	1.15	1.21	0.00
His	0.47	0.44	0.43	0.42	0.52	0.44	0.00
Ile	0.84	0.77	0.75	0.77	0.88	0.86	0.02
Leu	1.53	1.41	1.39	1.39	1.63	1.46	0.01
Lys	1.25	1.30	1.22	1.30	1.27	1.83	0.02
Met	0.40	0.38	0.39	0.36	0.44	0.47	0.00
Phe	1.12	0.94	0.95	0.94	1.07	0.87	0.01
Thr	0.82	0.80	0.74	0.79	0.83	0.98	0.00
Trp	0.27	0.25	0.24	0.24	0.28	0.29	0.04
Val	1.01	0.94	0.91	0.92	0.98	0.90	0.00

[1]HFB = high-fermentable high β-glucan hull-less barley; HFA = high-fermentable high amylose hull-less barley; MFB = moderate-fermentable hull-less barley; LFB = low fermentable hulled barley; LFW = low-fermentable wheat.

[2]Chemically-available Lys.

Table 3.4 Apparent ileal digestibility (AID), apparent total tract digestibility (ATTD), and hindgut fermentation of energy and nutrients of cereal grains[1] (DM basis)

| Item, % | Hull-less barley | | | Hulled barley | Wheat | | |
	HFB	HFA	MFB	LFB	LFW	SEM[2]	*P* value
GE							
AID	44.2[c]	49.1[c]	69.0[a,b]	66.4[b]	78.8[a]	2.91	<0.001
ATTD	86.9[a]	87.0[a]	89.5[a]	81.6[b]	88.5[a]	1.54	<0.001
ATTD – AID	40.5[a]	35.8[a]	18.2[b]	12.5[b]	6.26[b]	3.76	<0.001
DM							
AID	41.7[b]	47.2[b]	67.2[a]	64.0[a]	73.9[a]	2.87	<0.001
ATTD	87.4[b]	88.2[b]	90.5[a]	80.9[c]	87.7[b]	1.03	<0.001
ATTD – AID	45.7[a]	40.9[a]	23.5[b]	16.6[b]	13.9[b]	3.23	<0.001
CP							
AID	49.1[c]	52.1[c]	65.8[b]	66.8[b]	79.0[a]	3.37	<0.001
ATTD	75.9[c,d]	73.5[d]	80.5[b]	76.9[c]	87.0[a]	1.11	<0.001
ATTD – AID	25.8[a]	20.6[a,b]	13.6[b,c]	8.89[c]	7.14[c]	3.42	0.001
Starch							
AID	73.7[c]	69.6[c]	84.0[b]	92.1[a]	93.4[a]	1.69	<0.001
ATTD	99.8	99.7	99.7	99.8	99.8	0.05	0.383
ATTD – AID	26.0[a]	30.0[a]	15.6[b]	7.62[c]	6.26[c]	1.74	<0.001
β-glucan							
AID	19.4[b]	33.1[b]	66.0[a]	59.5[a]	70.2[a]	6.36	<0.001
ATTD	100.0[a]	100.0[a]	100.0[a]	99.9[a]	99.7[b]	0.03	<0.001
ATTD – AID	80.5[a]	66.9[a]	33.9[b]	40.4[b]	29.5[b]	6.35	<0.001

[a-d]Within a row, means without a common superscript differ (*P* < 0.05).

[1]HFB = high-fermentable high β-glucan hull-less barley; HFA = high-fermentable high amylose hull-less barley; MFB = moderate-fermentable hull-less barley; LFB = low-fermentable hulled barley; LFW = low-fermentable wheat.

[2]Based on n=6 observations per cereal grain.

Table 3.5 Standardized ileal digestibility (SID) of CP and AA in cereal grains[1] (DM basis)

| Item, % | Hull-less barley | | | Hulled barley | Wheat | | |
	HFB	HFA	MFB	LFB	LFW	SEM[2]	*P*-value
CP	63.0[c]	67.1[c]	83.1[b]	81.8[b]	89.1[a]	2.79	<0.001
Indispensable AA							
Arg	72.2[b]	73.6[b]	86.4[a]	86.1[a]	89.2[a]	2.24	<0.001
His	65.0[b]	68.3[b]	80.0[a]	79.7[a]	88.0[a]	3.48	<0.001
Ile	58.5[c]	60.6[c]	79.4[b]	78.8[b]	87.0[a]	1.93	<0.001
Leu	61.3[c]	63.6[c]	78.6[b]	78.2[b]	86.8[a]	2.38	<0.001
Lys	62.8[c]	70.0[b,c]	80.5[a]	79.6[a,b]	85.1[a]	3.33	<0.001
Met	62.0[c]	63.8[c]	77.3[b]	76.9[b]	85.9[a]	1.67	<0.001
Phe	62.5[c]	62.4[c]	79.0[b]	78.8[b]	87.5[a]	2.22	<0.001
Thr	53.5[b]	59.2[b]	77.7[a]	77.1[a]	81.9[a]	2.96	<0.001
Trp	69.3[c]	75.7[b]	88.8[a]	87.2[a]	86.9[a]	3.67	<0.001
Val	57.9[b]	60.6[b]	76.1[a]	76.0[a]	83.0[a]	2.87	<0.001
Dispensable AA							
Ala	50.6[b]	59.0[b]	73.0[a]	71.9[a]	80.1[a]	3.48	<0.001
Asp	50.5[b]	57.7[b]	72.9[a]	72.0[a]	78.5[a]	3.79	<0.001
Cys	48.4[b]	55.0[b]	72.5[a]	72.0[a]	80.7[a]	2.78	<0.001
Glu	68.2[c]	69.5[c]	86.1[b]	86.2[b]	92.9[a]	1.69	<0.001
Gly	53.5[b]	63.5[b]	85.4[a]	83.5[a]	89.9[a]	5.22	<0.001
Pro	88.5[c]	98.8[b]	116.0[a]	112.5[a]	114.8[a]	8.77	<0.001
Ser	59.5[b]	64.1[b]	80.5[a]	79.5[a]	86.5[a]	2.51	<0.001
Tyr	59.7[c]	63.3[c]	80.8[b]	80.1[b]	88.8[a]	2.46	<0.001
Total AA	64.6[c]	68.1[c]	83.9[a,b]	83.1[b]	90.0[a]	2.65	<0.001

[a-d]Within a row, means without a common superscript differ (*P* < 0.05).

[1]HFB = high-fermentable high β-glucan hull-less barley; HFA = high-fermentable high amylose hull-less barley; MFB = moderate-fermentable hull-less barley; LFB = low-fermentable hulled barley; LFW = low-fermentable wheat.

[2]Based on n=6 observations per ingredient.

Table 3.6 Energy metabolism of growing pigs according to diet and measured energy value of cereal grains[1]

	Hull-less barley			Hulled barley	Wheat		
Item	HFB	HFA	MFB	LFB	LFW	SEM[2]	*P*-value
FHP, Mcal	2.72	2.75	2.77	2.76	2.76	0.10	0.337
O_2 consumption, L/d	1003^b	1163^a	1098^{a,b}	1025^b	1163^a	104	0.045
CO_2 production, L/d	1180	1147	1134	1071	1085	44.8	0.145
CH_4, L/day	75.9^a	76.8^a	58.9^{a,b}	52.5^{a,b}	39.6^b	10.9	0.015
RQ	1.17^a	1.02^{a,b}	1.03^{a,b}	1.04^{a,b}	0.93^b	0.06	0.071
Heat Production, Mcal/d	5.24^b	5.80^a	5.54^{a,b}	5.19^{a,b}	5.74^a	0.22	0.036
Retained Energy, Mcal/d	2.28^a	1.42^b	1.66^{a,b}	1.57^b	1.66^{a,b}	0.15	<0.001
DE, Mcal/kg (as is)	3.58^a	3.56^a	3.59^a	3.29^b	3.52^a	0.02	<0.001
ME, Mcal/kg (as is)	3.21^{a,b}	3.14^b	3.23^{a,b}	3.01^c	3.28^a	0.06	<0.001
NE, Mcal/kg (as is)	2.12^a	1.76^b	1.98^{a,b}	1.91^{a,b}	1.94^{a,b}	0.10	0.011
ME:DE	0.90^{a,b}	0.88^b	0.90^{a,b}	0.91^{a,b}	0.92^a	0.02	0.018
NE:ME	0.66^a	0.56^b	0.61^{a,b}	0.64^{a,b}	0.59^{a,b}	0.03	0.016

$^{a-c}$Within a row, means without a common superscript differ ($P < 0.05$).

[1]HFB = high-fermentable high β-glucan hull-less barley; HFA = high-fermentable high amylose hull-less barley; MFB = moderate-fermentable hull-less barley; LFB = low-fermentable hulled barley; LFW = low-fermentable wheat; FHP, fasting heat production; RQ, respiratory quotient.

[2]Based on n=6 observations per ingredient.

Table 3.7 Calculated net energy of cereal grains[1]

| Mcal/kg (as is) | Hull-less barley | | | Hulled barley | Wheat | | |
	HFB	HFA	MFB	LFB	LFW	SEM[2]	*P*-value
NE from DE	2.55[a]	2.56[a]	2.57[a]	2.31[b]	2.53[a]	0.041	<0.001
NE from ME	2.38[a]	2.33[a,b]	2.40[a]	2.21[b]	2.44[a]	0.050	<0.001

[a-b]Within a row, means without a common superscript differ (*P* < 0.05).

[1]HFB = high-fermentable high β-glucan hull-less barley; HFA = high-fermentable high amylose hull-less barley; MFB = moderate-fermentable hull-less barley; LFB = low-fermentable hulled barley; LFW = low-fermentable wheat.

[2]Based on n=6 observations per ingredient.

A.

R² = 0.62; *P* < 0.001

HFB ◆HFA ▲MFB △LFB ○LFW

B.

R² = 0.67; *P* < 0.001

C.

R² = 0.70; *P* < 0.001

Diet β-glucan, % DM

R² = 0.20; *P* = 0.024
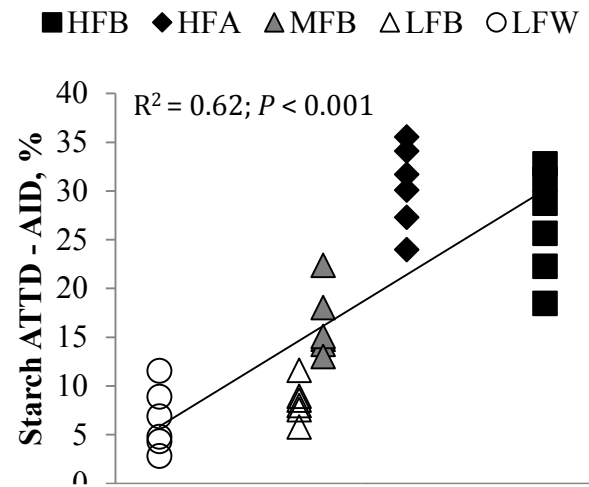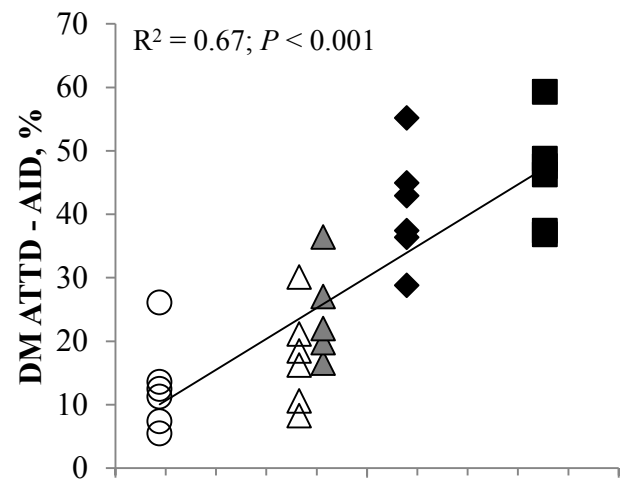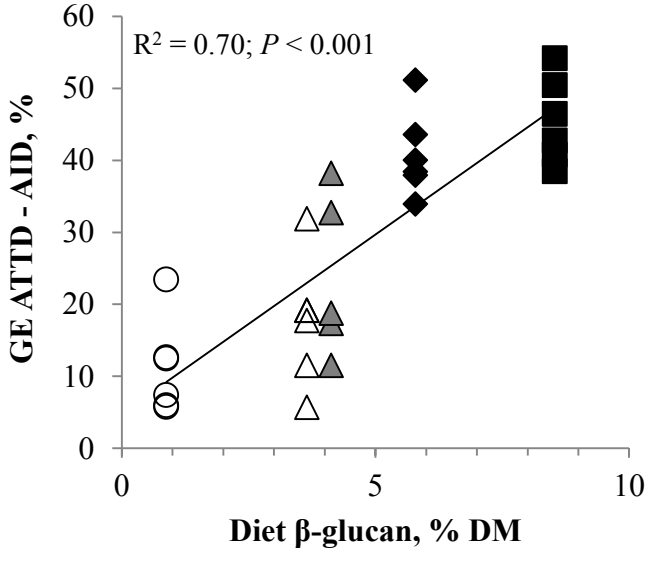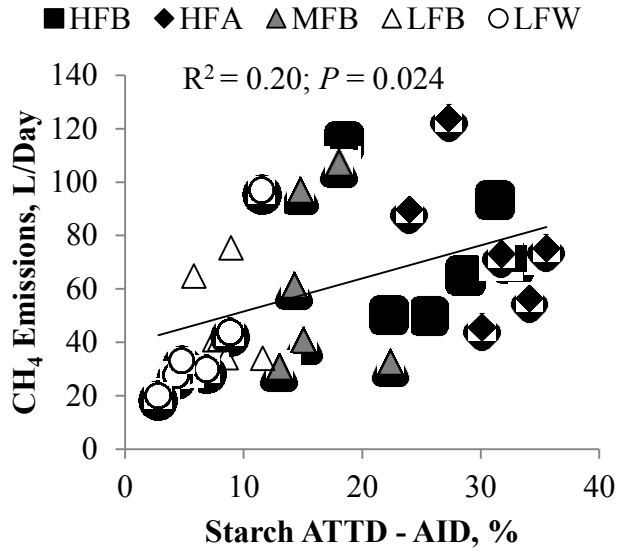
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Figure legends

Figure 3.1 Relationship between cereal grain β-glucan content and starch ATTD - AID (A), DM ATTD – AID (B), and GE ATTD – AID (C) in pigs consuming 5 cereal grains HFB, HFA, MFB, LFB, and LFW. HFB = high-fermentable high β-glucan hull-less barley; HFA = high-fermentable high amylose hull-less barley; MFB = moderate-fermentable hull-less barley; LFB = low-fermentable hulled barley; LFW = low-fermentable wheat.

Figure 3.2 Relationship between ATTD – AID of starch and CH_4 emissions per day in pigs consuming 5 cereal grains HFB, HFA, MFB, LFB, and LFW. HFB = high-fermentable high β-glucan hull-less barley; HFA = high-fermentable high amylose hull-less barley; MFB = moderate-fermentable hull-less barley; LFB = low-fermentable hulled barley; LFW = low-fermentable wheat.

Chapter 4. Whole grain composition modifies substrate availability in the hindgut for fermentation and thereby shifts faecal microbial profiles in pigs

4.1 Introduction

Dietary fibre, defined as the portion of carbohydrates resistant to mammalian digestion, is required for proper development and function of the gastrointestinal tract (GIT)[1]. Many functional properties of dietary fibre are related to microbial fermentation and colonization including GIT and immune development, nutrient metabolism and potential pathogen exclusion[2, 3]. Using semi-purified diets the GIT microbiota can be modulated through the inclusion of prebiotics[4] and specific fibrous compounds[5, 6]. For example, resistant starch (RS)[7-12], β-glucans[13, 14] and fermentable fibre[15] promoted proliferation and activity of commensal microbiota.

The fermentable fibre components studied in purified diets are naturally-occurring components in whole grains[16]. Conventionally, whole grains are the main source of energy for monogastrics and are thus a source of fermentable fibre. However, fermentable fibre that is consumed as part of a diet containing whole grains may have different physiological effects than purified fibre sources. Consumption of whole grains has been linked to positive manipulation of metabolic variables related to obesity[17], coronary heart disease[18] and type-2 diabetes[19] with changes attributed to host-microbial interactions[20]. However, the effect of whole grain composition on microbial composition has been overlooked until recently. Available dietary fermentable fibre and microbial composition are linked; thus, substrate flow into the hindgut for microbial fermentation may be a key determinant of microbial composition. However, information relating the

gastrointestinal tract (GIT) microbial composition to hindgut substrate availability has been ignored. Using an ileal cannulated pig model, we hypothesized that whole grains high in fermentable fibre, namely β-glucans, would increase hindgut substrate flow and availability thereby shifting the ileal and faecal microbial profiles in pigs. The objectives were to elucidate the effect of fermentable fibre composition of whole grains on substrate flow through the GIT and its impact on microbial and metabolite profiles in the foregut and hindgut.

4.2 Materials and methods

4.2.1 Whole grains and diets

Five whole grains were chosen based on chemical composition (Table 4.1) and potential fermentability (β-glucan content): high fermentable, high β-glucan hull-less barley (HFB, CDC-Fibar); high fermentable, high amylose hull-less barley (HFA, CDC-Hilose); moderate fermentable hull-less barley (MFB, CDC-McGwire); low fermentable hulled barley (LFB, Xena); and low fermentable hard red spring wheat (LFW, Utmost). As whole grain, HFB, HFA, MFB and LFW were obtained from the Crop Development Centre (Saskatoon, SK, Canada) and LFB was obtained from Viterra (Wetaskawin, AB, Canada). Grains were ground and included at 80% into experimental diets that met or exceeded nutrient requirements for growing pigs (Table 4.2)[21]. Diets contained 0.5 Cr_2O_3 as an indigestible marker to determine starch and dry matter (DM) flow and output.

4.2.2 Animals and design

The animal study was approved by the Animal Care and Use committee of the University of Alberta according to the guidelines of the Canadian Council on Animal Care and was conducted at the Swine Research and Technology Centre (Edmonton, AB, Canada)[22, 23]. A total of 7 crossbred castrated male pigs (initial BW of 27.7 ± 1.6 kg; Large White × Landrace) were surgically fitted with a T-cannula at the distal ileum as previously described[24, 25]. Pigs were housed individually in pens (1.2 m wide, 1.2 m long and 0.9 m high) in a thermo-controlled room (22 ± 2°C). The experiment was conducted as a 6 (period) × 5 (diet) Youden square design, obtaining 6 observations per whole grain. Pigs were allocated to the 5 whole grain diets in a completely randomized design. Daily feed allowance was set above maintenance requirement and fed in two equal daily feedings[21]. Pigs were adapted to test diets for 5 d with subsequent faeces and ileal digesta collection. Freshly voided faeces and ileal digesta were collected continuously as previously described[26, 27] and immediately frozen at -20°C.

4.2.3 Sample preparation and analysis

Prior to analyses, faeces and digesta were thawed, homogenized, subsampled and freeze-dried. Diets, whole grains, freeze-dried digesta and faeces were ground in centrifugal mill (Retch model ZMI; Brinkman Instruments, Rexdale, ON, Canada) through a 1-mm screen. Whole grains, digesta and faeces were analysed for DM by drying at 135°C in an airflow type oven for 2 h (method 930.15), acid detergent fibre (ADF) (method 973.18A-D), neutral detergent fibre (NDF) and total dietary fibre (TDF) were determined using enzymatic gravimetric methods[28, 29]. Whole grains, digesta and faeces were analysed for

Cr$_2$O$_3$ by spectrophotometry (model 80-2097-62, KBUltraspec III; Pharmacia, Cambridge, UK) at 440 nm after ashing at 450°C overnight[30]. Amylose (Amylose/Amylopectin Megazyme kit; Megazyme International, Bray, Ireland) β-Glucan (Mixed Linked β-glucan Kit; Megazyme International) and starch (Megazyme Total Starch kit; Megazyme International) were quantified with enzymatic methods. Short chain fatty acids (SCFA) in ileal digesta and faeces were quantified using gas chromatography as previously described[31]. Viscosity of whole grains was determined in duplicate at 0.5% (w/w) net β-glucan concentration by heating flour at 85°C for 1 h with a 0.1% v/w α-amylase addition on a rheometer at a shear rate of 12.9/s at 20°C with a 40 mm flat plate (Discovery HR-3 Hybrid Rheometer, TA Instruments, New Castle, DE, USA).

4.2.4 DNA extraction, 16S rRNA gene PCR and sequencing

Genomic DNA was extracted from ileal digesta and faeces of pigs using QIAamp® FAST DNA stool mini kit according to manufacturer's instructions (Qiagen, Valencia, CA, USA). The temperature was increased to 95°C to facilitate lysis of gram-positive bacteria. DNA concentrations were measured by Nano-Drop spectrophotometer system ND-1000 (Thermo Fisher Scientific Inc., Wilmington, USA), purity was assessed by determining the ratio of absorbance at 260 and 280 nm. DNA was submitted to the University of Minnesota Genomic Center for library preparation and sequencing on an Illumina MiSeq platform. Briefly, a PCR targeting V1-V3 regions of bacterial 16S rRNA genes was performed using the universal primers V1-forward Meta_V1_27F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**AGAGTTTGATCMTGGCT**

CAG) and V3-reverse Meta_V3_534R

(GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**ATTACCGCGGCTGCTG**
G). The bold part of each primer is complementary to eubacterial 16S sequences while upstream sequences corresponded to Ilumina adaptors required for sequencing and multiplexing. PCR was performed using KAPA HiFidelity Hot Start Polymerase with reaction times and cycling conditions of: 5 min at 95°C, 25 cycles of 20 s at 98°C, 15 s at 55°C, 1 m at 72°C, hold at 4°C. Subsequently, PCR products were diluted 1:100 and 5 μl used for a second PCR adding both the index and the flowcell adapters. The [i5] and [i7] refer to the index sequence codes used by Illumina with the p5 and p7 flow cell adapters in bold, forward –

(**AATGATACGGCGACCACCGA**GATCTACAC[i5]TCGTCGGCAGCGTC) and

reverse – (**CAAGCAGAAGACGGCATACGA**GAT[i7]GTCTCGTGGGCTCGG).

Cycling conditions were 5 min at 95°C, 10 cycles of 20 s 98°C, 15 s 55°C, 1 min 72°C, hold at 4°C. PCR products were pooled, size-selected and denatured with NaOH, diluted to 8 pM in Illumina's HT1 buffer, spiked with 20 PhiX and heat denatured at 96°C for 2 min prior to loading. A MiSeq 600 cycle v3 kit was used to sequence each sample. Nextera adapter sequences were used for run trimming.

4.2.5 Sequence data processing

Sequence data was analysed using a QIIME pipeline (MacQIIME 1.8.0 OS10.10)[32]. PANDAseq was used for quality filtering and to assemble the paired end reads into contigs with miscalled or uncalled bases discarded[33]. Resulting sequences were cleared of chimeras and singletons using UCHIME and UPARSE workflows, respectively, and

were subsequently clustered into operational taxonomic units (OTUs) having > 97% similarity with USEARCH[34-36]. Taxonomy was assigned using QIIME default setting, Ribosomal Database Project (RDP) classifier V2[37]. Alpha diversity and beta diversity estimations were conducted using the QIIME workflow core_diversity_analysis.py with a sampling depth of 49,000 and 1,800 for faeces and ileal digesta respectively with default parameters[38]. Briefly, alpha diversity was estimated using Whole Tree Phylogenetic Diversity (PD), Simpson and Shannon indices[39]. Differences in microbial communities between sample groups were investigated using phylogeny-based weighed UniFrac distance metric and to determine whether any whole grains caused significantly different bacterial communities with the analysis of similarities, ANOSIM, used on the weighted UniFrac distance matrix[40, 41].

4.2.6 Calculations

The following variables were calculated after the compositional analysis of whole grains, diet, ileal digesta and faeces. Ileal digesta flow of nutrients (mg/g DM fed) = [(100 − ileal digestibility of nutrient (%)) × total daily intake of nutrient (g) × 10]/ total daily DM intake (g). Faeces output of nutrient (mg/g DM fed) = [(100 − total tract digestibility of nutrient (%)) × total daily intake of nutrient (g) × 10]/total daily DM intake (g) as previously described[11].

4.2.7 Statistical analysis

Results are presented as means ± pooled SEM. Data were subject to analysis of variance using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) with whole grain as a

fixed variable and period and pig as random variables. Least squares means for each grain were reported. Multiple comparisons between least squares means were achieved using the PDIFF statement with TUKEY adjustment. To test the hypotheses, $P < 0.05$ was considered significant. The microbial community was analysed by subjecting genera level data to principal coordinates analysis (PCoA) with treatment groups as constraints using the Weighted UniFrac distance metric, followed by analysis of similarities (ANOSIM procedure). Principal component analysis (PCA) was performed using JMP software of SAS (version 8.0.2; SAS Institute). For PCA analysis, the loading plot of principle component 1 and principle component 2, the first 2 eigenvalues, were used to determine the correlation among cereal grain composition, ileal flow of starch (IF_Starch) and faecal output of DM (FO_DM), predominant bacterial phyla and faecal SCFA. The angles between the lines were used to describe the interrelationship. The raw sequences were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession no. SRP065675.

4.3 Results

4.3.1 Ileal substrate flow

Whole grains varied with respect to chemical composition and substrate flow (Table 4.1 and 4.3). Starch content ranged from 542 to 585 g/kg DM in hull-less barleys and was greater in LFB (627 g/kg DM) and LFW (610 g/kg DM). Amylose content was greatest in HFA (180 g/kg DM) and ranged between 7.3-123 g/kg DM for HFB, MFB, LFB and LFW. The β-glucan content ranged from 44.1 g/kg DM (LFB) to 103 g/kg DM (HFB) in the barley samples and was lowest in LFW (7.3 g/kg). The β-glucans in barley caused

viscosity to be greatest in all barley samples ranging between 303 and 342 mPa × s in HFB, HFA, MFB and LFB and lowest in LFW (0). The ADF content was greatest in LFB (94.0 g/kg DM) and ranged between 54.9-64.4 g/kg DM in HFB, HFA, MFB and LFW. The NDF was similar among grains ranging from 212 g/kg DM (MFB) – 263 g/kg DM (HFB).

Ileal starch flow was greatest ($P < 0.05$) in HFB and HFA and lowest ($P < 0.05$) in LFB and LFW. Faecal starch output did not differ ($P > 0.05$) among the whole grains. Ileal DM flow was greatest ($P < 0.05$) in HFB and HFA vs. MFB, LFB and LFW. However, faecal DM output was greatest ($P < 0.05$) in LFB and least ($P < 0.05$) in MFB. Ileal flow of β-glucan was greatest ($P < 0.05$) in HFB and least ($P < 0.05$) in LFW. Faecal β-glucan output did not differ ($P > 0.05$) among pigs consuming the whole grains.

4.3.2 Ileal and faecal microbial communities

Sequence data revealed whole grains had a measureable effect on microbial composition. Sequencing of 16S rRNA gene amplicons resulted in a total of 2,317,304 and 3,123,819 sequences in ileal digesta and faeces respectively. Samples with less than 1806 reads were excluded from the analysis leaving n = 4 for HFB, n = 5 for HFA, MFB, LFB and LFW for ileal digesta and n = 6 per whole grain for faeces. The sampling depth was set at 1,806 and 49,354 for ileal digesta and faeces samples respectively.

Firmicutes was the predominant phylum in ileal digesta of pigs, followed by Proteobacteria, Bacteroidetes and Actinobacteria (Table 4.4). Firmicutes was predominated by *Lactobacillus, Turicibacter, Sharpea* and *Clostridium* along with

unclassified genera of Peptostreptococcaceae and Clostridiaceae and an unclassified family in the Clostridales. Variation in whole grain composition did not affect ($P > 0.05$) ileal microbiota at the phylum level (Table 4.4) or genera level (Table 4.5). *Streptococcus* tended to increase ($P < 0.10$) in MFB compared to the other whole grains. Within the Proteobacteria, an unclassified genus of Enterobacteriaceae was predominant.

Firmicutes were the most abundant phylum in faeces, followed by Bacteriodetes, Tenericutes, Proteobacteria, Spirochaetes and Actinobacteria (Table 4.6). Firmicutes had increased ($P < 0.05$) abundance in HFB, HFA and MFB vs. LFB. In contrast, faecal abundance of Bacteroidetes increased ($P < 0.05$) in LFB vs. MFB. Within Firmicutes, an unclassified family and genus of Clostridiales, unclassified genus of Ruminococcaceae, Clostridiaceae and *Lactobacillus, Megasphaera, Streptococcus* and *Mitsuokella* were ubiquitous among faeces of pigs (Table 4.7). Faecal abundance of *Dialister* was greater ($P < 0.05$) in HFB, HFA and MFB vs. LFB and LFW. Faecal abundance of *Sharpea* tended to be greatest ($P < 0.10$) in HFB vs. LFB and LFW. Faecal abundance of an unclassified genus of Mogibacteriaceae and *Oscillospira* increased ($P < 0.05$) in LFB and LFW vs. HFB and HFA. Faecal abundance of *Phascolarctobacterium* was greatest ($P < 0.05$) in LFB vs. HFB, HFA and MFB. *Bulleidia* and an unclassified genus of Veillonellaceae increased ($P < 0.05$) in faecal abundance in HFA. Faecal abundance of *Ruminococcus* tended to increase ($P < 0.10$) in HFB vs. HFA, LFB and LFW. An unclassified genus of Lachnospiraceae increased ($P < 0.05$) in faecal abundance in LFB vs. all other whole grains. Within the phylum Bacteroidetes, unclassified genera of S24-7 predominated faeces of pigs consuming whole grains. An unclassified genus of P-2534-18b5 and an unclassified family and genus of Bacteroidales increased ($P < 0.05$) in faecal

abundance in LFB vs. HFB, HFA and MFB. Faecal abundance of *Prevotella* was greatest ($P < 0.05$) in LFB and least in MFB. *Parabacteroides* was greatest ($P < 0.05$) in LFB and LFW and least in HFA. Faecal abundance (0.83%) of *Akkermansia* increased ($P < 0.05$) in LFW vs. HFB, HFA and MFB and *Sphaerochaeta* tended to increase ($P < 0.10$) in LFW vs. HFA and MFB.

4.3.3 Ileal and faecal microbial diversity

Whole grains did not alter diversity in ileal digesta of pigs (Table 4.8). However, LFB increased ($P < 0.05$) faecal microbial diversity vs. HFA, as measured by whole tree PD, and is attributed to increased abundance and diversity of bacterial taxa within Bacteroidetes. Ileal and faecal phylogenetic composition was compared by using weighted UniFrac, a beta-diversity measure[41]. Based on the weighted UniFrac distance metric, ileal bacterial diversity did not differ (ANOSIM; $P > 0.05$). However, faecal bacterial diversity, measured by weighted UniFrac distance metric, indicated clustering of LFB (ANOSIM; $P < 0.05$) vs. other whole grains with a distance metric of 0.134 (Fig. 4.1.). Decreased ileal substrate flow of starch and fermentable fiber and increased ADF content from the barley hull likely altered type of substrate available for fermentation subsequently shifting microbial community structure.

4.3.4 PCA

The PCA of whole grain composition, ileal flow of starch and DM, faecal DM output and SCFA and predominant faecal bacterial phyla are shown in Fig. 4.2. The PC1 and PC2 explained 53.82 and 24.28% of variation, respectively. The loading plot indicates a

correlation of whole grain composition and specific bacterial phyla. Whole grain β-glucan and TDF content was closely correlated with ileal DM flow, faecal butyrate and propionate concentrations among component 1 and 2. Whole grain TDF and β-glucans content, ileal starch flow and Firmicutes were also positively affected by component 1; however, were separated by component 2. Additionally, Bacteroidetes were positively correlated with faecal output of DM and ADF along both components.

4.3.5 Ileal and fecal SCFA

Ileal acetate, propionate, branched chain fatty acids (BCFA) and total SCFA were greatest ($P < 0.05$) in HFA vs. other whole grains (Table 4.9). Caproic acid was greatest ($P < 0.05$) in HFA and least ($P < 0.05$) in MFB and LFB. Butyrate tended ($P < 0.10$) to be greater in MFB and LFB vs. other whole grains. Faecal SCFA concentrations did not differ ($P > 0.10$) among whole grains.

4.4 Discussion

This study determined how whole grain composition influences substrate flow and subsequent ileal and faecal microbial community composition. Different from previous studies that compared the microbial profiles before or after whole grain consumption, the present study compared the microbial profiles after consumption of whole grains differing in their chemical composition. The use of an ileal cannulated pig model allowed us to quantify the flow of substrate from the small intestine into the hindgut and thus to determine the availability of substrates for bacterial fermentation. Comparison of

microbial composition at various taxonomic levels revealed microbiota shifts as a result of whole grain composition and substrate flow.

4.4.1 Substrate flow

Diets high in RS or β-glucans can increase passage rate and substrate flow into the hindgut [7, 11, 15]. Similarly, whole grains with high viscosity, β-glucan (HFB) and RS (HFA) content in the present study increased ileal starch and DM flow into the hindgut, providing substrate for microbial fermentation. Increased DM output of pigs consuming LFB vs. other whole grains indicates the lower fermentability of substrates from LFB.

4.4.2 Ileal and fecal microbiota

In the present study Firmicutes dominated the ileal microbiota, with *Lactobacillus* prevailing as previously reported[11]. Similar to findings in the present study, dietary composition did not impact ileal microbiota[11]. Limited changes in ileal digesta microbial communities may be due to similar substrate abundance within the small intestine.

Firmicutes and Bacteroidetes dominated the pig faecal microbiota[43, 44]. These phyla are linked to whole grain consumption; however, little information exists linking chemical composition and substrate flow of whole grains to microbiota structure[20]. In the present study, faecal abundance of Firmicutes increased with ileal starch flow at the expense of Bacteroidetes in HFB, HFA and MFB. In contrast, decreased ileal starch flow in LFB increased faecal abundance of Bacteroidetes at the expense of Firmicutes. Previously, consumption of whole grains, specifically whole barley products, increased the Firmicutes to Bacteroidetes ratio[20, 45]. In the present study, decreased post-ileal

substrate flow increased abundance of Bacteroidetes, decreasing the Firmicutes to Bacteroidetes ratio in the hindgut. Low carbohydrate diets and diet-dependent reductions of Firmicutes are connected[46]. In the present study, reduction of ileal starch flow in LFB and LFW may mimic effects of a low carbohydrate diet, explaining the reduced abundance of Firmicutes. Reduced hindgut fermentation of carbohydrates may have health consequences related to reduced abundance of microbial butyrate producers and butyrate concentration[47].

Among Firmicutes, an unclassified genus of Ruminococcaceae was most abundant. Ruminococcaceae include genera negatively correlated with inflammatory markers[20]. Interestingly, pigs consuming HFB had increased faecal abundance of *Ruminococcus*. Increased ileal starch flow also increased the faecal abundance of genera *Dialister* and *Sharpea*, indicating a potential substrate preference of starch. Previously, *Dialister* increased after consumption of dietary fibre[48] and whole grains[20, 49].

Certain Firmicutes including *Phascolarctobacterium* were increased in pigs consuming low fermentable whole grains, LFB and LFW. Previously, dietary intervention with cruciferous vegetables, high in lignin and cellulose, increased abundance of *Phascolarctobacterium* in humans[50]. The low fermentable whole grains fed in the present study also had high acid detergent fibre (ADF), a measure of lignin and cellulose, indicating a potential substrate preference for members of *Phascolarctobacterium*. Faecal abundance of *Oscillospira* also increased in response to low fermentable whole grains, LFB and LFW vs. high fermentable whole grains, HFB and HFA. *Oscillospira* has been hypothesized to be part of a common core of microbiota[51] and presence of *Oscillospira* species may indicate a healthy GIT[52].

However, dietary interventions with fibre additives were inconsistent, with soluble corn fibre decreasing abundance of *Oscillospira*[48].

Bacteroidetes, including *Prevotella*, have been associated with consumption of non-starch polysaccharides[53] and fibre[54] and are carbohydrate and fibre degraders[55]. Abundance of Bacteroidetes may be related to composition and availability of specific substrates. Decreased ileal substrate flow and availability for fermentation in the hindgut and can shift microbiota to members that utilize endogenous host substrates including mucin, enzymes and luminal epithelial cells. Thus, lower ileal starch flow of pigs consuming LFW may increase faecal abundance of *Akkermansia*, demonstrating adaptability of the microbiota to utilize host substrates when dietary substrates are scarce. However, excessive degradation of mucin by taxa such as *Akkermansia* may decrease barrier function of the GIT potentially exposing the host to unwanted pathogens[56].

Responses of the microbiota to dietary intervention might be quick, with changes observable in less than one day[57-59]. In the present study, a short-term intervention was sufficient to elicit a faecal microbial shift. The whole grains in the present study differed only in their fibre composition indicating that the microbiota is very sensitive to changes in substrate structure and availability. The concept that each whole grain may have a unique composition matrix that will competitively favour specific bacteria is defined as a 'discrete structure'[57]. Our study indicates that this 'discrete structure' of whole grains influences substrate flow and availability thereby manipulating microbial composition.

4.3.4 Diversity

Compositional complexity of indigestible carbohydrates seems to be the driver of bacterial diversity[12, 60, 61] with whole grain barley, brown rice [20], legumes and vegetables[62] increasing microbial diversity. In the present study, faecal microbial diversity was only increased in pigs consuming LFB. Barley hulls, from LFB, are composed of ADF, recalcitrant lingo-cellulose, and are resistant to digestion and fermentation and can decrease small intestinal protein digestion. Consequently, pigs consuming LFB may have had increased flow and availability of ADF and protein for fermentation, potentially increasing cellulolytic and protein-utilizing bacteria explaining the increased diversity measured.

4.3.5 PCA

Clustering in the PCA supports the hypothesis that the composition of faecal abundance of Firmicutes was positively related to whole grain fermentable fibre composition and increased post-ileal substrate flow. The loading plot showed clustering of TDF and β-glucan content with ileal flow of DM and faecal propionate and butyrate. Promotion of hindgut SCFA production may benefit host gut health, because propionate can provide energy through gluconeogenesis and butyrate is the main energy source for colonocytes[63]. Furthermore, the strong correlation between Firmicutes and ileal flow of starch supports our hypothesis that substrate availability is an important determinant for microbial composition. Clustering of Bacteroidetes with ADF, the least fermentable portion of fibre, and faecal output of DM also points to the importance of substrate availability on resultant microbial profile.

4.3.6 Ileal and fecal SCFA

In the present study, increasing dietary amylose through the high amylose hull-less barley HFA increased foregut fermentation, evidenced by increased acetate, propionate and total SCFA concentrations in ileal digesta. Increasing dietary amylose previously increased net portal absorption of acetate, propionate and total SCFA[31]. Similarly, GIT propionate concentration increased when high amounts of amylose[10] and RS[9] were fed. Notably, concentrations of SCFA within digesta and faeces depends on production of SCFA by microbes and rate of absorption; therefore, digesta and faeces concentration of SCFA may not reflect actual microbial fermentation and SCFA production.

4.4 Conclusion

In conclusion, the present study provides insight on how whole grain composition can influence substrate flow and availability and thereby microbial composition. Several taxa of bacteria were associated with specific whole grains and substrate flow, offering evidence that gut bacteria can be modified rapidly. Due to the complex nature of whole grains, association of specific fibrous substrates to individual bacterial taxa remains difficult. Our study revealed that consumption of fermentable whole grains increased ileal starch flow, shifted faecal microbiota towards Firmicutes increasing *Dialister, Sharpea* and *Megasphaera*. Consumption of fermentable whole grains high in β-glucans and viscosity were strongly correlated with faecal propionate and butyrate and may promote health. Thus, dietary inclusion of whole grains rich in fermentable fibre should be

considered for future applications in pig nutrition to potentially correct or prevent dysbiosis.

4.5 References

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Table 4.1 Analysed composition of whole grains

Chemical composition (g/kg DM)	Hull-less barley			Hulled barley	Wheat
	HFB	HFA	MFB	LFB	LFW
DM	903	903	897	895	894
Starch	529	542	585	627	610
Amylose	2.0	180	114	112	123
TDF	224	184	146	170	141
ADF	54.9	61.7	61.7	94.0	64.4
NDF	263	238	212	247	259
β-glucan	103	74.2	49.2	44.1	7.3
Viscosity (mPa × s)	342	316	303	319	0

HFB, high fermentable, high β-glucan hull-less barley, CDC-Fibar; HFA, high fermentable, high amylose hull-less barley, CDC-Hilose; MFB, moderately fermentable barley, CDC-McGwire; LFB, low fermentable hulled barley, Xena; LFW, low fermentable hard red spring wheat, CDC-Utmost; DM, dry matter; ADF, acid detergent fibre; NDF, neutral detergent fibre.

Table 4.2 Diet composition of whole grain based diets on an as-fed basis

| Ingredient (g/kg, as fed) | Hull-less barley | | | Hulled barley | Wheat |
	HFB	HFA	MFB	LFB	LFW
Hulled barley	0.0	0.0	0.0	800	0.0
Hull-less barley	800	800	800	0.0	0.0
Wheat	0.0	0.0	0.0	0.0	800
Canola protein concentrate	72.2	72.2	72.2	72.2	72.2
Fishmeal	72.2	72.2	72.2	72.2	72.2
Canola oil	17.0	17.0	17.0	17.0	17.0
L-Lysine HCl	3.5	3.5	3.5	3.5	3.5
DL-Methionine	0.5	0.5	0.5	0.5	0.5
L-Tryptophan	0.3	0.3	0.3	0.3	0.3
Threonine	1.3	1.3	1.3	1.3	1.3
Limestone	6.0	6.0	6.0	6.0	6.0
Calcium phosphate	8.0	8.0	8.0	8.0	8.0
Salt	4.0	4.0	4.0	4.0	4.0
Vitamin premix*	5.0	5.0	5.0	5.0	5.0
Mineral premix†	5.0	5.0	5.0	5.0	5.0
Cr_2O_3	5.0	5.0	5.0	5.0	5.0

HFB, high fermentable, high beta-glucan hull-less barley, CDC-Fibar; HFA, high fermentable, high amylose hull-less barley, CDC-Hilose; MFB, moderately fermentable barley, CDC-McGwire; LFB, low fermentable hulled barley, Xena; LFW, low fermentable hard red spring wheat, CDC-Utmost.

*Provided the following per kilogram of diet: vitamin A, 8,250 IU; vitamin D3, 825 IU; vitamin E, 40 IU; 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; and vitamin B_{12}, 0.025 mg.

† Provided the following per kilogram of 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 $Ca(IO_3)_2$; and Se, 0.1 mg as Na_2SeO.

Table 4.3 Ileal substrate flow and fecal substrate output from ileal cannulated pigs fed 5 whole grains

Item	Hull-less barley			Hulled barley	Wheat		
	HFB	HFA	MFB	LFB	LFW	SEM	*P* value
Post-ileal flow (mg/g DMI)							
Starch	111[a]	127[a]	77[b]	38[c]	35[c]	6.93	<0.001
DM	544[a]	503[a]	348[b]	369[b]	291[b]	22.3	<0.001
β-glucan	69[a]	39[b]	14[c]	15[c]	3.0[d]	3.0	<0.001
Faecal output (mg/g DMI)							
Starch	0.6	0.8	0.7	0.5	0.5	0.2	0.484
DM	141[b]	135[b]	118[c]	192[a]	139[b]	3.24	<0.001

HFB, high fermentable, high β-glucan hull-less barley, CDC-Fibar; HFA, high fermentable, high amylose hull-less barley, CDC-Hilose; MFB, moderately fermentable barley, CDC-McGwire; LFB, low fermentable hulled barley, Xena; LFW, low fermentable hard red spring wheat, CDC-Utmost; DM, dry matter.
[a,b] Nonsimilar letters denote significant differences (*P* < 0.05) for each row by ANOVA.

Table 4.4 Bacterial phyla (relative abundance) in ileal digesta of pigs fed 5 whole grains as determined by 16S rRNA MiSeq Illumina Sequencing

| Phyla | Hull-less barley | | | Hulled barley | Wheat | | |
	HFB	HFA	MFB	LFB	LFW	SEM	*P* value
Firmicutes	91.5	98.1	93.5	79.3	71.6	10.7	0.130
Proteobacteria	7.50	0.0	5.60	19.2	26.8	10.8	0.155
Bacteroidetes	0.35	0.49	0.35	0.44	0.62	0.23	0.382
Actinobacteria	0.17	0.09	0.12	0.14	0.06	0.10	0.711

HFB, high fermentable, high β-glucan hull-less barley, CDC-Fibar; HFA, high fermentable, high amylose hull-less barley, CDC-Hilose; MFB, moderately fermentable barley, CDC-McGwire; LFB, low fermentable hulled barley, Xena; LFW, low fermentable hard red spring wheat, CDC-Utmost.

Table 4.5 Bacterial genera (relative abundance) in ileal digesta of pigs fed 5 whole grains as determined by 16S rRNA MiSeq Illumina Sequencing

| Phyla | Genera | Hull-less Barley | | | Hulled Barley | Wheat | | |
		HFB	HFA	MFB	LFB	LFW	SEM	P value
Firmicutes	*Lactobacillus*	32.3	27.8	5.87	24.8	20.4	13.2	0.681
	Turicibacter	26.7	33.5	43.6	23.1	34.0	11.9	0.670
	Clostridiaceae*	18.3	20.4	30.4	11.7	14.0	7.65	0.279
	Peptostreptococcaceae*	7.89	8.90	9.24	12.7	4.50	4.30	0.750
	Clostridiales†	2.11	2.71	2.69	2.16	1.48	0.978	0.804
	Sharpea	1.07	1.66	0.18	0.68	0.07	0.539	0.150
	Streptococcus	0.22	0.56	1.20	0.10	0.49	0.280	0.089
	Clostridium	0.07	3.32	0.65	0.03	0.17	1.36	0.385
Proteobacteria	Enterobacteriaceae*	11.3	2.09	2.90	19.1	21.4	11.6	0.149

HFB, high fermentable, high β-glucan hull-less barley, CDC-Fibar; HFA, high fermentable, high amylose hull-less barley, CDC-Hilose; MFB, moderately fermentable barley, CDC-McGwire; LFB, low fermentable hulled barley, Xena; LFW, low fermentable hard red spring wheat, CDC-Utmost.

* Unclassified genera of family.

† Unclassified family and genera of order.

Table 4.6 Bacterial phyla (relative abundance) in faeces of pigs fed 5 whole grains as determined by 16S rRNA MiSeq Illumina Sequencing

| Phyla | Hull-less barley | | | Hulled barley | Wheat | | |
	HFB	HFA	MFB	LFB	LFW	SEM	*P* value
Firmicutes	70.3[a]	65.8[a]	66.7[a]	51.7[b]	61.3[a,b]	3.57	0.011
Bacteroidetes	19.7[a,b]	21.5[a,b]	17.5[b]	30.7[a]	22.5[a,b]	2.96	0.029
Tenericutes	3.41	3.35	4.75	5.96	5.31	1.23	0.248
Proteobacteria	1.83	5.00	4.83	3.83	2.83	1.42	0.403
Spirochaetes	0.67	0.67	1.17	1.50	1.50	0.43	0.467
Actinobacteria	0.50	0.28	0.38	0.40	0.37	0.08	0.429

HFB, high fermentable, high β-glucan hull-less barley, CDC-Fibar; HFA, high fermentable, high amylose hull-less barley, CDC-Hilose; MFB, moderately fermentable barley, CDC-McGwire; LFB, low fermentable hulled barley, Xena; LFW, low fermentable hard red spring wheat, CDC-Utmost.

[a,b]Nonsimilar letters denote significant differences (*P* < 0.05) for each row by ANOVA.

Table 4.7 Bacterial genera (relative abundance) in faeces of pigs fed 5 whole grains as determined by 16S rRNA MiSeq Illumina Sequencing

Phyla	Genera	Hull-less barley			Hulled barley	Wheat		
		HFB	HFA	MFB	LFB	LFW	SEM	P value
Firmicutes	Ruminococcaceae[*]	21.0	17.8	19.8	17.7	18.0	2.61	0.803
	Clostridiales†	10.9	8.44	9.99	6.90	7.22	1.88	0.388
	Lactobacillus	10.2	7.12	7.41	5.94	16.7	4.37	0.436
	Dialister	3.59^a	6.16^a	3.23^a	0.07^b	0.31^b	1.81	0.040
	Sharpea	2.98 ^{a}	1.18 ^{a,b}	1.18 ^{a,b}	0.70 ^{b}	0.70^b	0.627	0.091
	Megasphaera	1.34	3.61	2.24	0.22	0.20	1.410	0.163
	Streptococcus	2.09	0.46	2.83	2.21	1.08	0.842	0.141
	Oscillospira	1.98^b	1.97^b	2.77^{a,b}	3.91^a	4.22^a	0.651	0.051
	Ruminococcus	2.93^a	1.24^b	1.80^{a,b}	1.11^b	1.18^b	0.505	0.063
	Mitsuokella	1.25	2.10	1.16	0.36	0.21	0.636	0.139
	Veillonellaceae[*]	1.60^b	4.25^a	1.19^b	0.33^b	0.52^b	0.793	<0.001
	Lachnospiraceae[*]	1.60^b	0.82^b	2.17^b	3.85^a	2.10^b	0.603	<0.001
	Clostridiaceae[*]	1.56	1.25	1.36	1.17	1.21	0.449	0.888
	Bulleidia	0.61^{a,b}	1.97^a	0.29^{ab}	0.12^b	0.16^{a,b}	0.430	0.034
	Mogibacteriaceae[*]	0.17^b	0.18^b	0.25^{ab}	0.40^a	0.38^a	0.065	0.041
	Phascolarctobacterium	0.08^c	0.12^{b,c}	0.38^{a,b,c}	0.75^a	0.61^{a,b}	0.124	0.002
Bacteroidetes	S24-7[*]	9.71	12.5	8.86	7.87	7.40	1.64	0.136
	p-2534-18b5[*]	2.29^b	1.75^b	1.78^b	7.30^a	4.00^{a,b}	1.59	0.081
	Prevotella	4.85^{a,b}	4.43^{a,b}	3.02^b	6.26^a	3.36^{a,b}	0.978	0.047
	Bacteroidales†	1.57^b	1.59^b	1.74^b	4.87^a	3.40^{a,b}	0.591	0.001
	Parabacteroides	0.38^{a,b}	0.28^b	0.55^{a,b}	1.06^a	1.11^a	1.85	0.009
Proteobacteria	Enterobacteriaceae[*]	0.47	0.47	3.42	0.96	1.12	0.873	0.110
Verrucomicrobia	*Akkermansia*	0.02^b	nd	nd	0.21^b	0.83^a	0.109	0.001
Spirochaetes	*Sphaerochaeta*	0.17^{a,b}	0.10^b	0.10^b	0.27^{a,b}	0.42^a	0.091	0.094

HFB, high fermentable, high β-glucan hull-less barley, CDC-Fibar; HFA, high fermentable, high amylose hull-less barley, CDC-Hilose; MFB, moderately fermentable barley, CDC-McGwire; LFB, low fermentable hulled barley, Xena; LFW, low fermentable hard red spring wheat, CDC-Utmost.

[a,b,c] Nonsimilar letters denote significant differences ($P < 0.05$) for each row by ANOVA.

* Unclassified genera of family.

[†]Unclassified family and genera of order.

Table 4.8 Ileal and faecal diversity indices of pigs fed 5 whole grains

| Diversity index | Hull-less barley | | | Hulled barley | Wheat | | |
	HFB	HFA	MFB	LFB	LFW	SEM	*P* value
Ileal							
Shannon	1.97	2.23	2.09	1.82	1.74	0.277	0.510
Simpson	0.59	0.64	0.62	0.53	0.49	0.083	0.550
Whole Tree PD	5.96	7.14	6.91	5.73	5.72	0.661	0.059
Faeces							
Shannon	6.33	5.94	6.69	7.33	6.73	0.390	0.200
Simpson	0.96	0.95	0.96	0.98	0.93	0.023	0.637
Whole Tree PD	29.7[a,b]	27.1[b]	35.2[a,b]	40.1[a]	34.1[a,b]	2.85	0.033

HFB, high fermentable, high β-glucan hull-less barley, CDC-Fibar; HFA, high fermentable, high amylose hull-less barley, CDC-Hilose; MFB, moderately fermentable barley, CDC-McGwire; LFB, low fermentable hulled barley, Xena; LFW, low fermentable hard red spring wheat, CDC-Utmost.
[a,b]Nonsimilar letters denote significant differences (*P* < 0.05) for each row by ANOVA.
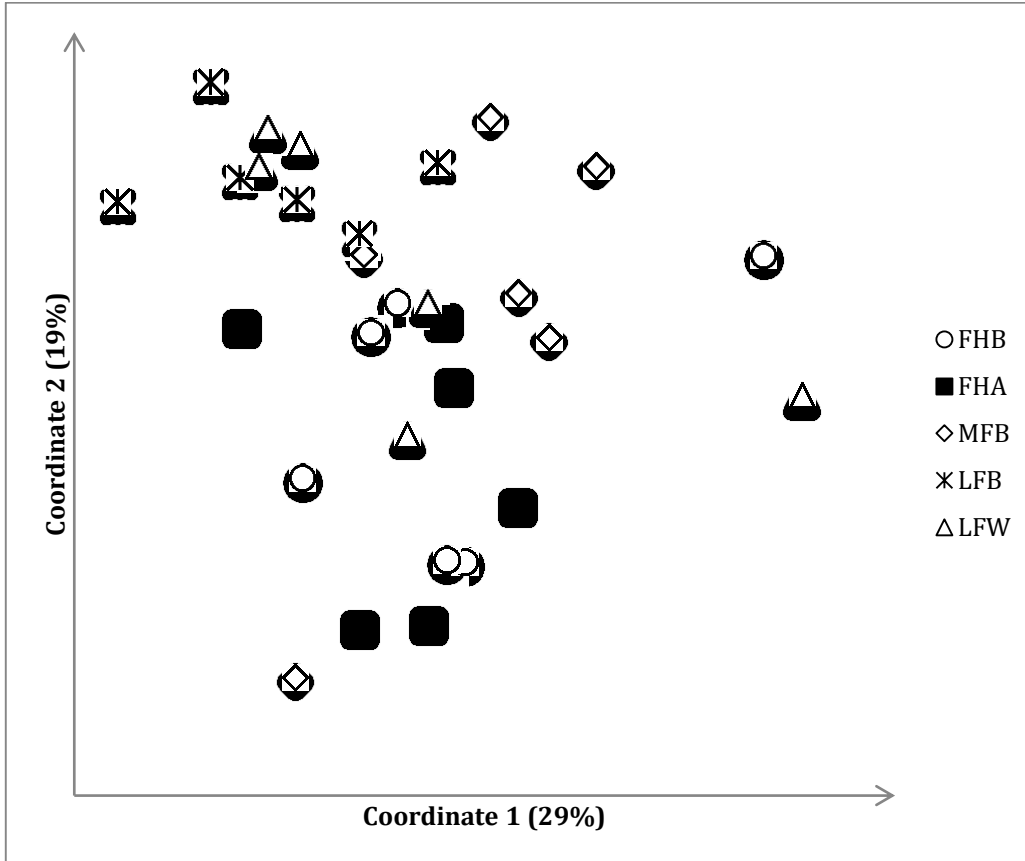
Figure 4.1 Principal Coordinate Analysis of weighted UniFrac distance metric. Pigs consuming the LFB diet were clustered ($P < 0.05$) distinct from pigs consuming the other 4 diets with a distance metric of 0.134.
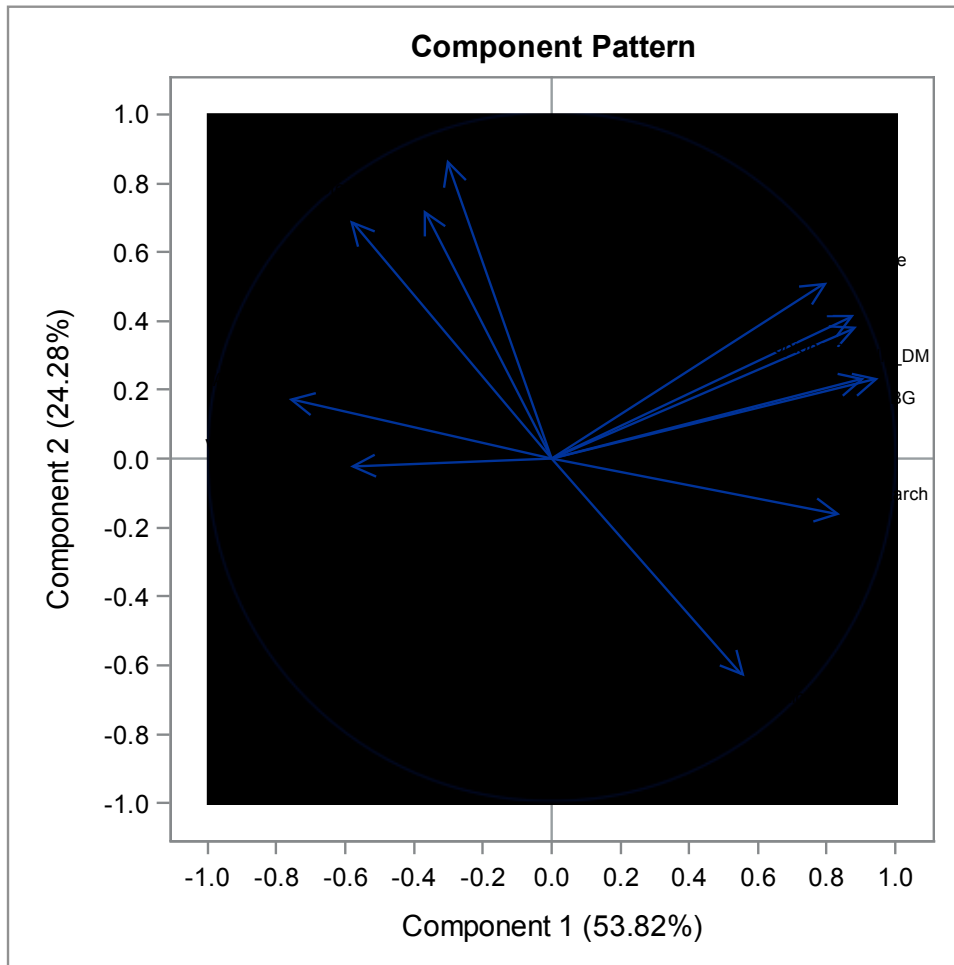
Figure 4.2 Loading plot showing the correlations among whole grain characteristics, abundant phyla, nutrient flow and microbial metabolites of the first 2 eigen values (Component 1 and Component 2) in pigs consuming 5 whole grains. Where, FO_DM = faecal output of DM g/kg DMI, IF_DM = ileal flow of DM g/kg DMI, IF_Starch = ileal flow starch g/kg DMI.

Table 4.9 Ileal and faecal SCFA concentration (umol/g wet weight)

Variable	Hull-less barley			Hulled barley	Wheat	SEM	P value
	HFB	HFA	MFB	LFB	LFW		
Ileal							
Acetate	18.0[b]	44.4[a]	21.1[b]	21.5[b]	24.3[b]	5.36	0.001
Propionate	2.42[b]	7.65[a]	2.32[b]	2.76[b]	2.68[b]	1.25	<0.001
Butyrate	0.83[b]	1.37[b]	2.11[a]	1.74[a]	1.27[b]	0.306	0.075
Caproic	0.13[a,b]	0.40[a]	0.12[b]	0.10[b]	0.16[a,b]	0.068	0.026
BCFA	0.88[b]	2.67[a]	1.18[b]	0.94[b]	1.36[b]	0.428	0.003
Total	22.2[b]	56.5[a]	26.8[b]	27.0[b]	29.6[b]	7.05	<0.001
Faecal							
Acetate	22.9	42.0	34.4	43.3	28.1	9.62	0.270
Propionate	11.6	9.73	9.75	10.0	8.61	1.82	0.790
Butyrate	6.56	4.67	4.41	5.26	4.08	1.07	0.454
Valeric	2.27	1.47	1.34	1.16	0.96	0.492	0.207
Caproic	0.38	0.37	0.35	0.40	0.29	0.109	0.942
BCFA	3.79	4.43	4.13	4.25	3.41	0.824	0.789
Total	46.4	62.3	54.5	64.1	54.9	9.87	0.765

HFB, high fermentable, high beta-glucan hull-less barley, CDC-Fibar; HFA, high fermentable, high amylose hull-less barley, CDC-Hilose; MFB, moderately fermentable barley, CDC-McGwire; LFB, low fermentable hulled barley, Xena; LFW, low fermentable hard red spring wheat, CDC-Utmost.

[a,b]Nonsimilar letters denote significant differences ($P < 0.05$) for each row by ANOVA.

Chapter 5. Chemical composition of whole grains alters viscosity and nutrient flow subsequently modifying microbiota profiles of young pigs regardless of protein quality

5.1 Introduction

When formulating diets for weaned pigs, nutritionists typically only consider the digestible nutrient content of feedstuffs and little consideration is made of how indigestible or fermentable nutrients may affect swine health or growth performance. Conventionally newly-weaned pigs are fed diets high in crude protein to ensure adequate growth rates. However, excess or undigested protein can be harmful by becoming a fermentative substrate for proteolytic bacteria. Proteolytic bacteria produce harmful metabolites that impair intestinal integrity, nutrient digestion and growth[1]. In young pigs, excess or fermentable protein can increase post-weaning diarrhoea[2] and decrease growth[3]. In humans, excess consumption of protein through red and processed meats may cause intestinal dysfunction and increase risk for colorectal cancer[4]. The damaging effects of protein fermentation is thought to begin with perturbations in gastrointestinal tract (GIT) microbial community structure[5] increasing the production of genotoxic metabolites, phenols, p-cresol, indoles, amines and ammonia, subsequently decreasing intestinal integrity and health[6].

Additional fibre may mitigate negative effects of protein fermentation by providing the microbiota an alternative fermentative substrate[1, 3, 7]. Inclusion of specific fermentable carbohydrates can decrease protein fermentation, ammonia and putrescine concentrations, shift microbial ecology and can increase animal growth [1, 3, 7, 8]. When fermentable carbohydrates are not limiting, ammonia and amines can be used as

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substrates for microbial protein synthesis. However, when fermentable carbohydrates are limiting undigested protein becomes an energy source yielding harmful compounds[3,9]. Microbial and metabolite modifications in the GIT due to fibre additives may be responsible for mitigating consequences of protein fermentation[5]. Fermentation of fibre by specific microbial species results in the production of short-chain fatty acids (SCFA). In particular, the SCFA butyrate is a preferred energy substrate for colonoctyes promoting normal epithelial differentiation, integral for colonic health[10]. Low or limiting luminal SCFA, from low fermentable carbohydrate diets, are thought to contribute to cellular atrophy and inflammation of the GIT[5]. In colitis models, resistant starch (RS) consumption may play a protective role due to increased luminal SCFA concentrations and mucus production[11].

Although dietary fermentable carbohydrates can mitigate some consequences of protein fermentation, the use of whole grains as a source of fermentable carbohydrates has been overlooked until recently[12, 13]. Conventional cereal grains such as wheat and barley are commonly used in swine diets as sources of energy but may also be a source of fermentable carbohydrates. Barley β-glucans, a naturally occurring fermentable carbohydrate, is known to increase digesta viscosity. Inclusion of barley into the diet may favour the growth performance of weaned pigs by increasing digesta viscosity and retention time, improving nutrient digestibility and providing an alternative fermentative substrate to protein. Evidence suggests that high amylose barley, rich in non-starch polysaccharides (NSP), such as β-glucans, can manipulate the intestinal microbial ecosystem and their secondary metabolites[14,15]. Different cultivars of hull-less barley can decrease relative proportions of *Salmonella*[16] and affect host response dependant upon

grain composition, with amylose content increasing luminal butyrate production and β-glucan content increasing luminal SCFA concentrations[14,16]. Our objectives were to evaluate the interaction between whole grains (wheat (W), digestible hull-less barley (dHB) and fermentable hull-less barley (fHB)) and digestible (SBM) vs. heat-damaged (autoclaved SBM) protein on nutrient flow and digestibility, microbial and metabolite profiles. We hypothesized that fermentable whole grains would increase post-ileal nutrient flow and retention time, increasing nutrient digestibility, subsequently shifting microbial and metabolite profiles, mitigating negative effects of protein fermentation.

5.2 Materials and methods

5.2.1 Animals and diets

The animal study was approved by the University of Alberta Animal Care and Use Committee for Livestock, followed the guidelines of the Canadian Council on Animal Care and was conducted at the Swine Research and Technology Centre (Edmonton, AB, Canada)[17]. A total of 36 crossbred weaned barrows (initial BW of 7.7 ± 1.0 kg Duroc sire × Large White/Landrace F_1, Genex Hybrid, Hypor, Edmonton, AB, Canada) were assigned to 1 of 6 diets in a 2×3 factorial design. Pigs were weaned and group housed at 21 d of age to 25 d of age (d 5) to acclimatize to solid feed. On d 5, pigs were selected for a mid range body weight and transferred to individual metabolism pens for a 2-day adaptation to test diets and pens. Individual pens were 120×40 cm allowing freedom of movement, visual contact with other pigs and free access to drinking water. Temperature was maintained at 22°C with a 12 h photoperiod.

As whole grain, fermentable hull-less barley (fHB, CDC Hilose), digestible hull-less barley (dHB, CDC McGwire) and 1 low-fermentable Canadian Western red spring wheat (W, CDC Utmost) were obtained from the Crop Development Centre (Saskatoon, SK, Canada) (Table 5.1). Six diets were formulated to provide regular SBM or heat-damaged aSBM in combination with W, dHB or fHB (Table 5.2). The SBM was heat-damaged by steam autoclaving at 121°C for 20 min. Diets were formulated to meet or exceed nutrient requirements of young pigs (Table 5.2)[18]. Supplemental dietary AA were used to balance diets for standardized ileal digestible lysine, threonine, methionine and tryptophan. Diets contained TiO_2 as an indigestible marker for determination of apparent ileal and total tract nutrient digestibility.

5.2.2 Sample collection

Pigs were acclimatized to a starter diet from d 0 to d 5, followed by 50% replacement of the starter diet by experimental diets on d 6. On d 7, 100% experimental diets were fed for 18 days at 3 × the maintenance energy requirement via bi-daily feedings at 0800 and 1600 h, respectively[18]. Leftover and wasted feed was weighed back 1 h post-prandially to determine average daily feed intake (ADFI). Pigs were weighed on d 7, d 11, d 18 and d 25 to determine average daily gain (ADG). Subsequently, ADFI and ADG were used to calculate feed efficiency (gain/feed). On d 18 freshly voided faeces were collected using plastic bags and immediately frozen for nutrient digestibility and microbial analysis. On d 25, pigs were euthanized to collect digesta from ileum and colon. Four hours post-prandial, pigs were sedated with 16 mg/kg body weight (BW) of ketamine HCl (Ketalar, Bioniche, Belleville, ON, Canada), 2.2 mg/kg BW of xylazine (Rompun, Bayer, Toronto,

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ON, Canada) and 8 mg/kg BW of azaperone (Stresnil, Elanco, Guelph, ON, Canada) prior to killing with intracardial injection of 106 mg/kg BW of sodium pentobarbital (Euthanyl, Biomeda, Cambridge, ON, Canada) and exsanguination. Pigs were placed in dorsal recumbency, a midline abdominal incision was made to expose internal body cavity and intestinal segments were clamped and dissected from the mesentery. Ileum and colon digesta samples were snap frozen in liquid N_2 and stored at -80°C for SCFA and microbial analysis. A 3×3 cm section of duodenum, jejunum and ileum were sampled, washed in cold saline and fixed in 10% formalin at room temperature. The formalin was replaced after 48 h for histology analysis.

5.2.3 Sample preparation and chemical analysis

Prior to analyses, faeces and digesta were freeze-dried. Diets, whole grains, freeze-dried ileal digesta and faeces were ground in a centrifugal mill (Retch model ZMI; Brinkman Instruments, Rexdale, ON, Canada) through a 1-mm screen. The whole grains, diets, digesta and faeces were analysed for dry matter (DM) (method 930.15), gross energy using an adiabatic bomb calorimeter (model 5003; Ika-Werke GMBH & Co. KG, Staufen, Germany) and crude protein (CP) (method 984.13A-D). Amylose according to manufacturers instructions (Amylose/Amylopectin Kit; Megazyme International, Bray, Ireland), total starch (Megazyme Total Starch kit; Megazyme International, Bray, Ireland), β-glucan (Mixed Linked β-glucan Kit; Megazyme International, Bray, Ireland) and total dietary fibre (TDF) were analysed based on enzymatic analysis (methods 996.11, 995.16 and 985.29, respectively)[19], Whole grains and diets were further analysed for acid detergent fibre (method 973.18A-D) and neutral detergent fibre (NDF)[20] at the

Agricultural Experiment Station Chemical Laboratories, University of Missouri–Columbia[19]. Diets and digesta were analysed for amino acids (method 982.30E) and chemically-available Lysine (method 975.44) and whole grains and diets for ether extract (EE; method 920.39A)[19]. Experimental diets, digesta and faeces were all analysed for TiO_2 content. Concentration of SCFAs in colonic digesta was determined with the use of GC as previously described[21]. Viscosity of whole grain W, dHB and fHB was determined using a rheometer at a shear rate of 12.9/s and a temperature of 20°C as previously described[22].

5.2.4 Histology

Two transverse sections of duodenum, jejunum and ileum per pig were stained with the hematoxylin-eosin and analysed with the use of a Nikon image analyser. Twenty well-oriented villi and crypts were identified and measured.

5.2.5 DNA extraction, 16S rRNA gene PCR and sequencing

Genomic DNA was extracted from colonic digesta and faeces of pigs using QIAamp® FAST DNA stool mini kit according to manufacturer's instructions (Qiagen, Valencia, CA, USA). A temperature of 95°C was used to facilitate lysis of gram-positive bacteria. Resulting DNA concentrations were measured by a Nano-Drop spectrophotometer system ND-1000 (Thermo Fisher Scientific Inc., Wilmington, DE, USA) with purity assessed by determining the ratio of absorbance at 260 and 280 nm. DNA was submitted to the University of Minnesota Genomic Center for library preparation and sequencing on an Illumina MiSeq platform targeting V1-V3 regions of

bacterial 16S rRNA genes previously described in Chapter 4. Breifly, PCR was

performed using universal primers V1-forward Meta_V1_27F

(TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAGTTTGATCMTGGCTC

AG) and V3-reverse Meta_V3_534R

(GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTACCGCGGCTGCTGG).

PCR with KAPA HiFidelity Hot Start Polymerase with reaction times and cycling

conditions of: 5 min at 95°C, 25 cycles of 20 s at 98°C, 15 s at 55°C, 1 m at 72°C and

hold at 4°C. Resulting PCR products were diluted and used for a second PCR adding both

index and flowcell adapters. The PCR products were pooled, size-selected, diluted and

spiked with 20 PhiX and heat denatured at 96°C for 2m prior to loading.

5.2.6 Sequence data processing

Sequence data was analysed using a previously described QIIME pipeline (Chapter

4) (MacQIIME 1.8.0 OS10.10)[23]. Breifly, PANDAseq was used for quality filtering and

assembling paired end reads[24]. Sequences were cleared of chimeras and singletons and

clustered into operational taxonomic units (OTUs) haing 97% similarity using

USEARCH software[25-27]. Ribosomal Database Project (RDP) classifier V2[28] was used

to assign taxonomy. Diversity analysis was conducted using a QIIME workflow

core_diversity_analysis.py with a sampling depth of 749 and 1806 for colon digesta and

faeces, respectively [29, 30]. Phylogeny-based weighed UniFrac distance metric was used to

estimate community relationships[31,32].

5.2.7 Calculations

Apparent ileal digestibility (AID) and apparent total tract digestibility (ATTD) of nutrients were calculated for each pig based on the correction of TiO_2 content with the following equation:

$$AID \text{ or } ATTD \,(\%) = \{1-[(T_d/T_{f/i})/(N_d/N_{f/i})]\} \times 100$$

where T_d and $T_{f/i}$ are TiO_2 content of the diets and faeces or ileal effluent, respectively, and N_d and $N_{f/i}$ are the nutrient contents in the diets and faeces or ileal effluent, respectively. Hindgut fermentation of DM and starch in the large intestine was calculated by subtraction of ATTD – AID. Ileal digesta flow of DM and starch was calculated for each pig with the use of the following equation:

$$\text{Ileal flow (mg/g DMI)} = [(100 - AID \% \text{ of nutrient}) \times \text{total daily intake of nutrient g} \times 10]/ DMI \,(g)$$

Faecal output of nutrients was calculated with the use of the following equation:

$$\text{Fecal output (mg/g DMI)} = [(100 - ATTD \% \text{ of nutrient}) \times \text{total daily intake of nutrient in g} \times 10]/ DMI \,(g)$$

5.2.8 Statistical analysis

Results are presented as means ± pooled SEM. The mixed procedure of SAS was used to analyse data (SAS Inst. Inc., Cary, NC, USA) with experimental diets as the fixed effect and pig and period as random effects. Least squares means for grain, protein and grain × protein were reported. Multiple comparisons between least squares means were achieved using the PDIFF statement with TUKEY adjustment. Significance was declared as $P < 0.05$. The microbial community was analysed by subjecting genera level data to

Principal Coordinate Analysis (PCoA) with the three whole grains as constraints using the Weighted UniFrac distance metric, followed by analysis of similarities (ANOSIM procedure). Principal Component Analysis (PCA) was performed using JMP software of SAS (version 8.0.2; SAS Institute). The loading plot of principle component 1 and principle component 2 were used to determine the correlation among cereal grain composition, post-ileal flow of β -glucan (IF_BG) and faecal output of DM (FO_DM) and predominant bacterial phyla.

5.3 Results

5.3.1 Whole grain composition

Hull-less barley grains were regarded as digestible vs. fermentable based on their amylose and β-glucan content. Mixed linked β-glucan content ranged from 7.3 in W to 74.2 g/kg in fHB (Table 5.1). This increased β-glucan content in dHB and fHB raised subsequent dietary β-glucan content (Table 5.2). Cereal grain viscosity ranged from 0 in W to 316 mPa × s in dHB. Starch content ranged from 542 in fHB to 610 g/kg in W. Amylose content ranged between 114 in dHB to 180 g/kg in fHB. Chemically-available lysine was lower in diets containing heat-damaged protein (aSBM), indicating autoclaving was sufficient to decrease protein quality.

5.3.2 Growth performance

Throughout the study, pigs readily consumed diets without incidence of diarrhoea. Feed intake and gain decreased ($P < 0.05$) in aSBM vs. SBM, but efficiency was not affected (Table 5.3). Daily gain increased ($P < 0.05$) in fHB vs. W and efficiency was

greater ($P < 0.05$) in dHB and fHB vs. W, irrespective of protein source (aSBM vs. SBM).

5.3.3 Nutrient flow and digestibility

Post-ileal flow of DM, DE, CP and starch did not differ ($P > 0.05$) among diets (Table 5.4). Post-ileal flow of β -glucan increased ($P < 0.05$) in fHB vs. dHB and W, attributable to the higher β -glucan content of fHB. Faecal output of DM increased ($P < 0.05$) and faecal output of CP decreased ($P < 0.05$) in W vs. dHB and fHB. The AID of DM, DE, CP, starch or β-glucan did not differ ($P > 0.05$) among cereal grains or protein sources. The AID of Phe was greatest ($P < 0.05$) in W vs. fHB. The AID of remaining AA did not differ ($P > 0.05$) among cereal grains or protein sources (Table 5.3). The ATTD of DM was greatest ($P < 0.05$) and the ATTD of CP was least ($P < 0.05$) in dHB and fHB vs. W.

5.3.4 Effect of whole grain on colon digesta and fecal microbial communities

Sequencing of 16S rRNA gene amplicons resulted in a total of 397416 and 867350 sequences (mean of 11039 and 24093 per sample) for colonic digesta and faeces, respectively. Samples with less than 750 reads were excluded from the analysis leaving n = 5 per experimental treatment for colonic digesta and $n = 6$ per experimental treatment for faeces. The sampling depth was set at 750 and 1800 for colonic digesta and faeces samples respectively.

Firmicutes was the predominant phylum in colonic digesta of pigs, followed by Bacteroidetes, Tenericutes, Proteobacteria, Actinobacteria and Spirochaetes (Table 5.5).

Colonic abundance of Firmicutes increased ($P < 0.05$) with fHB vs. W. The phylum *Actinobacteria* and unclassified genus of Coriobacteriaceae was greatest ($P < 0.05$) in fHB vs. W and dHB. Phylum Spirochaetes, genus *Tremponema*, unclassified genus of Bacteroidales, genus *p-75-a5* of Erysipelotrichaceae and the unclassified family of RF39 increased ($P < 0.05$) in abundance in W vs. dHB and fHB (Table 5.6). The relative abundance of *Mitsuokella,* an unclassified genus of Veillonellaceae and Succinivibrio increased ($P < 0.05$) in fHB vs. W. *Roseburia* increased ($P < 0.05$) in dHB vs. W.

In faeces, the predominant phyla were Firmicutes and Bacteroidetes (Table 5.7). Firmicutes increased ($P < 0.05$) in abundance in SBM vs. aSBM. Faecal abundance of Proteobacteria increased ($P < 0.05$) in fHB vs. W. Faecal abundance of Tenericutes and Spirochaetes were greatest ($P < 0.05$) in W vs. fHB and dHB respectively. Faecal abundance of *Prevotella* increased ($P < 0.05$) in dHB vs. W (Table 5.8). Faecal abundance of the unclassified family and genus of S25-7, Lachnospiraceae and an unclassified family of RF39 increased ($P < 0.05$) in W vs. fHB. The unclassified genus of Clostridiaceae also increased ($P < 0.05$) in faecal abundance in W vs. dHB and fHB. Faecal abundance of Coriobacteriaceae and *Ruminococcus* was greatest ($P < 0.05$) in fHB vs. dHB and W. Faecal abundance of *Mitsuokella*, unclassified genus of Veillonellaceae, *Dialister, Megasphaera* and *Succinivibrio* had increased faecal abundance ($P < 0.05$) in fHB vs. W.

5.3.5 Microbial diversity

Microbial diversity in colonic digesta or faeces did not differ ($P > 0.05$) between SBM vs. aSBM (Table 5.9). Colonic digesta microbial diversity increased ($P < 0.05$) in W vs.

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dHB and fHB as measured by the Shannon index. Colonic digesta and faeces microbial diversity increased ($P < 0.05$) in W vs. fHB, as indicated by Simpson and Shannon indices respectively. Colonic and faecal phylogenetic composition was compared using weighted UniFrac, a beta diversity measure[31, 32]. The weighted UniFrac distance metric indicated there was clustering of the fHB vs. W and dHB (ANOSIM; $P < 0.05$) in faeces, with a distance metric of 0.424 (Fig. 5.1).

5.3.6 PCA

The PCA of whole grain composition, ileal flow of β-glucan, faecal DM output and predominant faecal bacterial phyla are shown in Fig. 5.2. Principle components 1 and 2 explained 42 and 20% of variation, respectively. The loading plot indicates a correlation of whole grain composition, digesta flow and predominating phyla. Whole grain viscosity and ileal flow of β-glucan was closely correlated with Proteobacteria among components 1 and 2. Whole grain amylose and TDF content was also positively correlated to Actinobacteria by component 1, however, was slightly separated by component 2. Faecal output of DM was positively correlated to Tenericutes and Spirochaetes by both components.

5.3.7 Microbial metabolites

Colonic concentrations of acetate, isobutyrate and isovalerate did not differ ($P > 0.05$) among grain or protein sources (Table 5.10). A grain × protein interaction occurred causing colonic propionate concentration to be greater ($P < 0.05$) in fHB/SBM diet vs. W/SBM, W/aSBM and fHB/aSBM diets. The interaction between grain × protein also

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caused colonic butyrate and total SCFA concentration to be greater ($P < 0.05$) in the fHB/SBM vs. W/SBM and fHB/aSBM diets.

5.4 Discussion

5.4.1 Animal performance

The present study determined how whole grain grains, differing in their composition, interact with digestible (SBM) vs. heat-damaged protein (aSBM) on growth performance, nutrient flow and digestibility and colonic and faecal microbial community composition in newly weaned pigs. Inclusion of aSBM as a heat-damaged protein source decreased gain, in agreement with a previous study feeding fermentable protein[3]. The negative growth response to aSBM was likely due to decreased intake, indicating a potential palatability issue for aSBM. Similar to previous work, dietary inclusion of fermentable carbohydrates, fHB vs. W, increased gain of young pigs[3].

5.4.2 Substrate flow and nutrient digestibility

Interestingly, hull-less barley varieties, dHB and fHB, increased feed efficiency vs. W, attributable to the increased ATTD of DM. The increased ATTD of DM for dHB and fHB vs. W is likely due to changes in digesta retention time explainable by the greater β-glucan content of the hull-less barley grains. High RS, β-glucans or viscous fibre can increase substrate flow into the hindgut for fermentation[33, 34] and increase nutrient digestibility due to prolonged GIT retention time[34, 35]. Thus, increased post-ileal β-glucan flows of dHB and fHB vs. W may have increased digesta viscosity and hindgut retention time, allowing for complete enzymatic hydrolysis and microbial fermentation.

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Increased DM output of W vs. dHB and fHB, indicates nutrients are escaping digestion, explaining the lower ATTD of DM. Fermentable fibre such as β-glucan can increase endogenous N loss[36], increase microbial protein synthesis and N excretion in faeces, explaining the lower ATTD of CP and increased CP output observed for dHB and fHB vs. W.

5.4.3 Colon and fecal microbiota

In the present study Firmicutes and Bacteroidetes dominated the pig colonic and faecal microbiota, as previously reported[37-40]. Colonic abundance of Firmicutes and Actinobacteria increased in dHB and fHB vs. W, in agreement with previous dietary inclusion of barley products[41]. Increased post-ileal flow of β-glucan in dHB and fHB vs. W likely increased the amount of fermentable substrate available for colonic enrichment of Firmicutes and Actinobacteria. Lower ileal flow of β-glucan in W may have mimicked the effect of low carbohydrate diets, previously reducing Firmicutes abundance[42] and butyrate-producing bacteria[43]. Colonic abundance of *Roseburia* was greater in dHB vs. W, in agreement with human dietary interventions with whole grain barley[12, 41] and soluble corn fibre[44]. Colonic enrichment of *Roseburia* may be beneficial to gut health as members of this genus are known butyrate producers[45]. Dietary carbohydrate: protein ratios have previously been studied in pigs and other species to help mitigate consequences of protein fermentation[1, 46]. Concluded a positive effect, kittens consuming a moderate carbohydrate: moderate protein diet vs. low carbohydrate: high protein diet had increased abundance of Veillonellaceae[46]. Thus, increased colonic and

faecal abundance of Veillonellaceae in fHB vs. W may indicate a desirable carbohydrate: protein ratio.

Inclusion of aSBM decreased abundance of Firmicutes and tended to enrich Bacteroidetes in faeces. Previously, protein enriched weight loss diets reduced members of Firmicutes in humans[47]. The shift in faecal microbiota from Firmicutes towards Bacteroidetes with inclusion of aSBM does not however imply an undesirable outcome. In particular, perturbations in small intestinal histology were not observed with dietary inclusion of heat-damaged protein (aSBM) (Table 5.11). Similarly, dietary fermentable protein shifted microbial composition[1] and changed expression of tight junction proteins[48]; however, undesirable changes in intestinal barrier function have yet to be observed[48].

Increased colonic and faecal abundance of lactate producer *Mitsuokella* may have increased faecal abundance of lactate utilizer, *Megasphaera* in fHB vs. W. *Megasphaera* is a major butyrate producer, promoting intestinal health[49]. The cross talk and analogous increase in *Mitsuokella* and *Megaspheara* has previously been cited[46]. Increased post-ileal β-glucan flow in dHB and fHB vs. W increased faecal abundance of *Dialister,* indicating their substrate preference for fermentable carbohydrates. Abundance of *Dialister* is positively correlated to dietary fibre[44] and whole grain consumption[12]. Fermentable barley, fHB vs. W, had increased faecal abundance *Ruminococcus*, in agreement with previous dietary interventions with barley products[41] and RS[47]. Increased amylose in fHB vs. W may have caused the increase in *Ruminococcus,* as members display amylolytic activity[50]. *Ruminococcus* may benefit the host, as members of Ruminococcaceae have been negatively correlated with inflammatory markers[12].

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Flow of β-glucan into the hindgut was met with increased faecal abundance of *Proteobacteria* in fHB vs. W. Members of the Proteobacteria may prefer fibre substrates, including β-glucan, as crude fibre and ADF digestibility have been positively correlated with this phylum[39]. *Tenericutes* are positively correlated with crude fibre digestibility in pigs[39]. Interestingly, even though W did not have increased flow of fibre to the hindgut, *Tenericutes* had increased abundance vs. fHB.

Results of the weighted UniFrac show that fermentable hull-less barley, fHB, altered faecal microbial community structure, shown by clustering away from dHB and W. Alpha diversity analysis revealed that fHB changed the abundance of community members rather than changing community composition, as fHB had lower colonic and faecal diversity vs. W. Interestingly, dietary intervention with RS in humans decreased alpha diversity of microbiota vs. a high NSP or a weight loss diet[51]. The homogenous nature of amylose in fHB vs. W may support enrichment of specific microbial taxa, as previously proposed by Salonen et al. (2014). The clustering of fHB, which was high in amylose and β-glucans, within the PCA may indicate importance of fiber type on microbial community structure. This supports our hypothesis that composition of faecal microbial communities are distinctly different based on whole grain composition and subsequent post-ileal nutrient flow.

5.4.4 Metabolites of fermentation

Studies have shown that digesta SCFA concentration is a result of both carbohydrate and N availability for microbes[3, 52]. The interaction between grain and protein on SCFA concentrations may have been due differences in N and carbohydrate

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availability among the experimental diets. Butyrate concentration was greatest in digesta of pigs consuming the fHB/SBM experimental diet, demonstrating that fHB fed with SBM provided an ideal ratio of fermentable carbohydrates with available N, allowing proliferation of butyrate producing bacteria. Butyrate is the preferred energy substrate for colonocytes, promoting normal differentiation, imperative for gut health[10]. Characteristically excess or undigestible protein is deaminated and decarboxylated by microbes yielding putrefactive compounds[53] or BCFA[1, 7]. However, microbes can utilize excess or undigestible protein to synthesize new microbial protein when sufficient fermentable carbohydrates are available[9]. Although protein quality was decreased by autoclaving SBM as evidenced by lower available lysine levels in aSBM, the aSBM did not increase protein fermentation. This indicates that fermentable carbohydrates found in the whole grains, W, dHB and fHB, may have been adequate to mitigate excess protein fermentation, but allow for synthesis of microbial protein. In particular, excess CP output in dHB and fHB provide some evidence of microbial protein synthesis. Our results indicate there may be an optimum balance between protein and fermentable carbohydrates in pig diets, as previously proposed[3].

5.5 Conclusion

In conclusion, the present study provides insight on how whole grain composition can influence post-ileal substrate flow, nutrient digestibility, microbial and metabolite profiles and growth performance in newly weaned pigs. Pigs consuming fHB vs. W had enriched colonic Firmicutes and shifted faecal and colonic microbiota towards fibre-degrading microbial taxa, Veillonellaceae, *Mitsuokella* and *Succinivibrio*. High β-glucan

content in dHB and fHB increased viscosity vs. W, subsequently increasing digesta

retention, ATTD of DM, improving growth performance and feed efficiency.

Interestingly, inclusion of heat-treated protein, aSBM, only had inconsequential effects

on feed intake and microbial composition. In conclusion, fermentable whole grains are

beneficial to the weaned pig by changing how substrates flow through the GIT,

improving digestion capabilities, microbial and metabolite profiles and growth

performance, regardless of protein quality.

5.6 References

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Table 5.1 Analyzed composition of cereal grains

Items	W	dHB	fHB
Chemical composition (g/kg DM)			
DM	894	897	903
GE (MJ/kg)	18.8	18.8	19.1
CP	197	162	167
EE	0.0	1.7	17.7
Starch	610	585	542
Amylose	123	114	180
TDF	141	146	184
ADF	64.4	61.7	61.7
NDF	259	212	238
β-glucan	7.3	49.2	74.2
Viscosity (mPas × s)	0	316	303

W, wheat; dHB, digestible hull-less barley; fHB, fermentable hull-less barley; DM, dry matter; GE, gross energy; CP, crude protein; EE, ether extract; TDF, total dietary fibre; ADF, acid detergent fibre; NDF, neutral detergent fibre.

Table 5.2 Ingredient and nutrient composition of the cereal grain based diets with autoclaved or untreated soybean meal

Items	SBM			aSBM		
	W	dHB	fHB	W	dHB	fHB
Ingredients (g/kg as-fed)						
Wheat HRS	693	-	-	693	-	-
Barley - McGwire	-	670	-	-	670	-
Barley - Hilose			670			670
SBM	200	200	200	-	-	-
aSBM[*]	-	-	-	200	200	200
Herring meal	50.0	50.0	50.0	50.0	50.0	50.0
Canola oil	15.5	35.5	35.5	15.5	35.5	35.5
Limestone	10.0	10.0	10.0	10.0	10.0	10.0
Mono/dical phoshate	3.0	4.3	4.3	3.0	4.3	4.3
Salt	5.0	5.0	5.0	5.0	5.0	5.0
L-Lysine HCl	3.0	3.3	3.3	3.3	3.6	3.6
L-Threonine	0.8	1.3	1.3	0.9	1.4	1.4
DL-Methionine	0.4	0.6	0.6	0.4	0.7	0.7
L-Tryptophan	0.1	0.5	0.5	0.2	0.5	0.5
Vitamin premix†	5.0	5.0	5.0	5.0	5.0	5.0
Mineral Premix‡	5.0	5.0	5.0	5.0	5.0	5.0
Choline Chloride	0.3	0.3	0.3	0.3	0.3	0.3
Celite	5.0	5.2	5.2	4.5	4.7	4.7
TiO_2	4.0	4.0	4.0	4.0	4.0	4.0
Chemical composition (g/kg DM)						
DM	911	914	919	913	922	918
GE (MJ/kg)	18.7	19.1	19.1	18.8	19.1	19.6
Starch	431	434	372	434	424	380
CP	294	262	265	280	251	260
EE	38.5	67.2	67.4	31.9	58.9	65.4
NDF	105	995	112	110	121	182
ADF	38.0	28.3	27.3	43.0	29.6	31.7
β-glucan	6.4	34.8	50.1	6.0	35.7	51.7
Ash	69.5	69.6	67.9	68.0	64.7	67.8
Chemically-available Lys	15.7	15.2	15.4	14.0	13.9	14.9

SBM, soybean meal; aSBM, autoclaved SBM; W, wheat; dHB, digestible hull-less barley; fHB, fermentable hull-less barley; GE, gross energy; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre.

[*]Steam autoclaved at 121°C for 20 min.

†Provided the following per kilogram of diet: vitamin A, 8,250 IU; vitamin D_3, 825 IU; vitamin E, 40 IU; 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; and vitamin B_{12}, 0.025 mg.

‡ Provided the following per kilogram of 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 $Ca(IO_3)_2$; and Se, 0.1

mg as Na_2SeO_3.

Table 5.3 Growth performance, apparent ileal digestibility (AID), and apparent total tract digestibility (ATTD) of nutrients in pigs fed cereal grain based diets with autoclaved or untreated soybean meal

Item	SBM			aSBM			SEM	P values		
	W	dHB	fHB	W	dHB	fHB		Grain	Protein	G × P
Growth performance										
ADG (kg/d)	0.40[b]	0.44[a,b]	0.46[a]	0.38[b]	0.40[a,b]	0.41[a]	0.014	0.010	0.003	0.411
ADFI (kg/d)	0.53	0.53	0.56	0.51	0.50	0.50	0.019	0.730	0.009	0.604
Efficiency (gain/feed)	0.75[b]	0.82[a]	0.83[a]	0.76[b]	0.80[a]	0.82[a]	0.022	0.009	0.754	0.806
AID (%)										
DM	59.7	65.3	55.5	58.3	67.5	60.5	4.35	0.111	0.579	0.752
DE	61.1	68.4	58.6	59.2	69.1	64.4	4.77	0.129	0.674	0.682
CP	67.8	71.2	67.7	66.4	70.6	70.9	3.86	0.631	0.897	0.821
Starch	77.7	87.8	75.9	78.5	89.0	81.4	5.35	0.119	0.571	0.890
β-glucan	58.2	58.1	30.3	35.1	35.0	34.9	10.41	0.356	0.155	0.363
Indispensible AA										
Chemically-available Lys	0.84	0.83	0.80	0.74	0.84	0.82	0.025	0.221	0.258	0.069
Arg	0.84	0.82	0.81	0.82	0.83	0.82	0.020	0.750	0.871	0.711
His	0.83	0.79	0.77	0.78	0.80	0.77	0.024	0.434	0.619	0.395
Ile	0.82	0.77	0.74	0.79	0.79	0.76	0.022	0.107	0.930	0.553
Leu	0.82	0.78	0.75	0.80	0.79	0.77	0.022	0.082	0.796	0.613
Lys	0.85	0.83	0.80	0.73	0.84	0.81	0.024	0.212	0.245	0.070
Met	0.84	0.83	0.80	0.82	0.85	0.82	0.021	0.368	0.559	0.465
Phe	0.82[a]	0.77[a,b]	0.74[b]	0.81[a]	0.79[a,b]	0.76[b]	0.023	0.033	0.668	0.791
Thr	0.77	0.75	0.72	0.71	0.78	0.77	0.025	0.614	0.920	0.104
Trp	0.85	0.85	0.82	0.80	0.87	0.86	0.018	0.157	0.804	0.069
Val	0.85	0.85	0.82	0.80	0.87	0.86	0.018	0.157	0.804	0.069
ATTD (%)										
DM	81.8[b]	84.5[a]	83.7[a]	81.4[b]	84.1[a]	83.5[a]	0.600	<0.001	0.436	0.968
DE	83.1	84.8	83.6	82.8	84.1	83.8	0.712	0.083	0.599	0.774
CP	84.1[a]	79.3[b]	78.3[b]	82.6[a]	77.9[b]	77.2[b]	1.43	<0.001	0.221	0.989

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SBM, soybean meal; aSBM, autoclaved SBM; W, wheat; dHB, digestible hull-less barley; fHB, fermentable hull-less barley; G, grain; P, protein.

[a, b]No G×P interaction, main effect of grain means separation shown. Means within a row without a common superscript differ, $P < 0.05$.

Table 5.4 Post-ileal flow and faecal output of nutrients in pigs fed cereal grain based diets with autoclaved or untreated soybean meal

Items	SBM			aSBM			SEM	P value		
	W	dHB	fHB	W	dHB	fHB		Grain	Protein	G × P
Post-ileal flow (mg/g DMI)										
DM	402.9	347.2	455.3	417.1	325.3	394.9	44.0	0.110	0.579	0.752
DE (kcal/g DMI)	1741	1445	1894	1829	1407	1665	198	0.134	0.716	0.729
CP	138.9	124.7	120.2	145.7	124.6	110.6	16.7	0.289	0.943	0.887
Starch	54.9	53.1	89.7	93.2	46.5	70.6	16.1	0.144	0.752	0.211
β-glucan	2.09^c	22.6^b	34.9^a	3.67^c	28.3^b	33.6^a	3.39	<0.001	0.457	0.542
Faecal output (mg/g DMI)										
DM	181.7^a	155.0^b	165.0^b	185.0^a	158.3^b	163.3^b	5.75	<0.001	0.686	0.847
DE (kcal/g DMI)	753.2	693.3	739.9	769.3	721.6	748.0	34.2	0.218	0.488	0.947
CP	68.2^b	89.6^a	79.9^{a,b}	73.8^b	93.2^a	86.8^{a,b}	6.04	0.003	0.227	0.954

SBM, soybean meal; aSBM, autoclaved SBM; W, wheat; dHB, digestible hull-less barley; fHB, fermentable hull-less barley; G, grain; P, protein.

[a, b] No G×P interaction, main effect of grain means separation shown. Means in a row with superscripts without a common letter differ, $P < 0.05$.

Table 5.5 Bacterial phyla (relative abundance) in colon digesta of pigs consuming cereal grain based diets with autoclaved or untreated soybean meal as per 16S rRNA Illumina MiSeq sequencing

Phyla	SBM			aSBM				P values		
	W	dHB	fHB	W	dHB	fHB	SEM	Grain	Protein	G × P
Firmicutes	59.2^b	64.5^{a,b}	77.4^a	60.7^b	71.1^{a,b}	69.5^a	4.82	0.035	0.983	0.342
Bacteroidetes	21.6	25.4	15.2	25.0	17.3	16.9	4.38	0.262	0.781	0.385
Tenericutes	7.54	3.70	3.36	6.10	4.24	4.43	1.40	0.080	0.960	0.649
Proteobacteria	2.54	2.15	1.85	0.83	1.38	1.68	0.835	0.940	0.211	0.657
Actinobacteria	0.24^b	0.39^b	1.10^a	0.21^b	0.53^b	3.77^a	0.661	0.005	0.096	0.092
Spirochaetes	2.11^a	0.39^b	0.09^b	1.90^a	0.30^b	0.68	0.513	0.003	0.804	0.683

SBM, soybean meal; aSBM, autoclaved SBM; W, wheat; dHB, digestible hull-less barley; fHB, fermentable hull-less barley; G, grain; P, protein.

[a, b] No G × P interaction, main effect of grain means separation shown. Means in a row with superscripts without a common letter differ, $P < 0.05$.

Table 5.6 Bacterial genera (relative abundance) in colonic digesta of pigs fed cereal grain based diets with autoclaved or untreated soybean meal as per 16S rRNA Illumina Miseq

Phyla	Genera	SBM			aSBM			SEM	P values		
		W	dHB	fHB	W	dHB	fHB		Grain	Protein	G × P
Actinobacteria	Coriobacteriaceae*	0.20^b	0.24^b	1.00^a	0.18^b	0.42^b	3.62^a	0.642	0.005	0.092	0.100
Bacteroidetes	S24-7*	7.51	13.91	6.37	11.96	11.18	6.97	3.09	0.194	0.764	0.520
	Prevotella	4.50	4.93	7.21	3.27	3.18	8.15	2.78	0.332	0.769	0.880
	Bacteroidales†	2.86^a	1.10^b	0.75^b	2.77^a	0.99^b	0.64^b	0.435	<0.001	0.767	0.999
	p-2534-18B5*	2.01	2.41	0.04	3.19	0.84	0.12	1.26	0.160	0.924	0.556
Firmicutes	*Lactobacillus*	10.3	17.7	32.5	15.9	23.9	19.7	5.43	0.079	0.947	0.169
	Ruminococcaceae*	15.4	17.3	10.1	16.6	12.7	16.6	2.59	0.604	0.650	0.127
	Clostridiales†	8.28	7.43	4.84	7.04	7.54	7.82	1.75	0.722	0.670	0.488
	Lachnospiraceae*	6.45	2.29	2.42	4.07	2.68	1.28	1.48	0.070	0.400	0.650
	p-75-a5	3.37^a	0.38^b	0.14^b	2.37^a	0.61^b	0.18^b	0.556	<0.001	0.601	0.502
	Ruminococcus	1.57	2.39	1.3	1.87	0.92	2.59	0.795	0.928	0.954	0.244
	Clostridiaceae*	2.49	1.37	0.45	0.97	1.45	1.49	0.691	0.560	0.821	0.201
	Oscillospira	1.56	1.93	1.43	1.37	1.31	2.14	0.529	0.835	0.945	0.457
	Coprococcus	0.82	0.78	0.31	1.08	1.12	0.63	0.311	0.240	0.249	0.992
	Megasphaera	0.14	2.33	4.31	0.35	5.70	1.39	1.82	0.128	0.882	0.250
	Dialister	0.22	0.52	7.09	0.52	3.87	3.85	2.34	0.116	0.943	0.396
	Mitsuokella	0.13^b	$0.52^{a,b}$	4.22^a	0.58^b	$1.37^{a,b}$	1.24^a	0.893	0.039	0.454	0.088
	Peptostreptococcaceae*	0.77	0.38	0.19	1.40	0.09	0.20	0.593	0.262	0.814	0.734
	Christensenellaceae*	0.54	0.46	0.18	1.88	0.68	0.45	0.367	0.065	0.056	0.249
	Veillonellaceae*	0.08^b	$1.21^{a,b}$	3.11^a	0.03^b	$0.65^{a,b}$	0.77^a	0.500	0.004	0.025	0.080
	Roseburia	0.07^b	1.49^a	$0.92^{a,b}$	0.32^b	2.00^a	$0.34^{a,b}$	0.577	0.039	0.919	0.643
Proteobacteria	Enterobacteriaceae*	1.77	0.57	0.59	0.46	0.33	0.75	0.523	0.643	0.436	0.578
	Succinivibrio	0.06^b	$0.56^{a,b}$	1.10^a	0.13^b	$0.52^{a,b}$	0.86^a	0.258	0.009	0.752	0.835

Spirochaetes	*Treponema*	1.41[a]	0.34[b]	0.07[b]	1.70[a]	0.25[b]	0.61[b]	0.421	0.009	0.483	0.762
Tenericutes	RF39†	7.54[a]	3.69[b]	3.36[b]	6.10[a]	4.22[b]	4.43[b]	1.40	0.080	0.961	0.649

SBM, soybean meal; aSBM, autoclaved SBM; W, wheat; dHB, digestible hull-less barley; fHB, fermentable hull-less barley.

[a, b]No G×P interaction, main effect of grain means separation shown. Means in a row with superscripts without a common letter differ, $P < 0.05$.

*Unclassified genera of family.

† Unclassified family and genera of order.

Table 5.7 Bacterial phyla (relative abundance) in faeces of pigs fed cereal grain based diets with autoclaved or untreated soybean meal as per 16S rRNA Illumina MiSeq sequencing

Phyla	SBM			aSBM				P values		
	W	dHB	fHB	W	dHB	fHB	SEM	Grain	Protein	G × P
Firmicutes	46.0[a]	42.7[a]	46.7[a]	36.3[b]	39.2[b]	40.0[b]	4.07	0.731	0.023	0.658
Bacteroidetes	36.3	43.8	38.2	48.2	44.5	42.2	4.27	0.599	0.097	0.357
Proteobacteria	4.33[b]	5.50[a,b]	10.67[a]	4.50[b]	8.33[a,b]	9.83[a]	2.17	0.038	0.686	0.686
Tenericutes	5.67[a]	3.67[a,b]	1.17[b]	4.67[a]	3.17[a,b]	2.17[b]	1.01	0.006	0.841	0.592
Spirochaetes	3.00	1.00	1.17	1.83	1.50	1.67	0.540	0.081	0.901	0.221

SBM, soybean meal; aSBM, autoclaved SBM; W, wheat; dHB, digestible hull-less barley; fHB, fermentable hull-less barley; G, grain; P, protein.

[a, b]No G × P interaction, main effect of grain mean separation shown. Means in a row with superscripts without a common letter differ, $P < 0.05$.

Table 5.8 Bacterial genera (relative abundance) in faeces of pigs fed cereal grain based diets with autoclaved or untreated soybean meal experimental diets as per 16s rRNA Illumina Miseq

Phyla	Genera	SBM			aSBM			SEM	P values		
		W	dHB	fHB	W	dHB	fHB		Grain	Protein	G × P
Actinobacteria	Coriobacteriaceae*	0.11^b	0.10^b	0.93^a	0.10^b	0.18^b	1.04^a	0.299	0.010	0.801	0.980
Bacteroidetes	S25-7*	12.9^a	11.1^{a,b}	10.7^b	15.2^a	5.07^{a,b}	7.30^b	2.68	0.054	0.258	0.248
	Prevotella	8.12^b	22.5^a	20.6^{a,b}	16.8^b	28.1^a	26.1^{a,b}	5.04	0.035	0.120	0.936
	Bacteroidales†	4.55	4.09	2.44	3.85	3.56	3.80	0.889	0.358	0.948	0.352
	P-2534-18B5	3.51	0.48	0.57	5.24	3.14	0.91	1.78	0.130	0.274	0.796
Firmicutes	Rumminococcaceae*	14.0	13.6	10.5	12.2	8.38	9.44	2.02	0.276	0.100	0.534
	Clostridiales†	7.78	5.38	5.19	6.32	7.23	3.91	1.65	0.312	0.828	0.537
	Lactobacillus	4.98	3.47	4.52	1.99	3.52	3.00	1.44	0.976	0.217	0.581
	Lachnospiraceae*	3.52^a	1.13^{a,b}	0.35^b	3.65^a	1.35^{a,b}	0.55^b	1.01	0.012	0.828	0.999
	Christensenellaceae*	2.71	1.31	0.73	0.88	1.12	0.36	0.552	0.093	0.088	0.279
	Oscillospira	2.53	1.67	1.41	1.67	1.24	1.07	0.389	0.088	0.097	0.783
	Clostridiaceae*	1.57^a	0.71^b	0.32^b	1.22^a	0.69^b	0.44^b	0.284	0.004	0.717	0.684
	Roseburia	0.90	1.07	0.50	0.29	1.37	0.44	0.379	0.124	0.688	0.481
	Ruminococcus	0.77^b	0.96^b	2.55^a	0.59^b	0.63^b	1.32^a	0.423	0.007	0.086	0.376
	Mitsuokella	0.47^b	0.43^{a,b}	0.86^a	0.19^b	0.42^{a,b}	1.30^a	0.275	0.021	0.830	0.431
	Veillonellaceae*	0.17^b	2.73^{a,b}	6.75^a	1.12^b	4.17^{a,b}	4.40^a	1.338	0.004	0.990	0.317
	Dialister	0.16^b	0.68^{a,b}	5.02^a	0.04^b	1.99^{a,b}	4.66^a	1.451	0.008	0.814	0.812
	Megasphaera	0.15^b	2.66^{a,b}	2.89^a	0.96^b	1.68^{a,b}	2.65^a	0.867	0.043	0.847	0.591
Proteobacteria	*Succinivibrio*	0.60^b	3.87^{a,b}	9.35^a	0.66^b	5.53^{a,b}	5.89^a	1.72	0.001	0.670	0.296
	Enterobacteriaceae*	3.37	0.81	0.86	3.16	2.08	3.60	1.75	0.338	0.213	0.489
Spirochaetes	*Treponema*	2.50	0.84	0.99	1.90	1.30	1.63	0.514	0.085	0.691	0.435
Tenericutes	RF39†	5.67^a	3.16^{a,b}	1.27^b	4.37^a	3.18^{a,b}	2.08^b	0.981	0.007	0.848	0.560

SBM, soybean meal; aSBM, autoclaved SBM; W, wheat; dHB, digestible hull-less barley; fHB, fermentable hull-less barley; G, grain; P, protein.

[a,b]No G × P interaction, main effect of grain means separation shown. Means in a row with superscripts without a common letter differ, $P < 0.05$.

*Unclassified genera of family.

† Unclassified family and genera of order.

Table 5.9 Alpha diversity indices in colonic and fecal digesta of pigs fed fed cereal grain based diets with autoclaved or untreated soybean meal

Item	SBM			aSBM			SEM	P values		
	W	dHB	fHB	W	dHB	fHB		Grain	Protein	G × P
Colonic digesta										
Shannon*	6.47^a	5.73^b	4.89^b	6.09^a	5.31^b	5.53^b	0.319	0.009	0.204	0.837
Simpson*	0.97^a	0.94^{a,b}	0.88^b	0.95^a	0.90^{a,b}	0.93^b	0.023	0.063	0.185	0.759
Faeces										
Shannon*	6.45^a	6.26^{a,b}	5.65^b	6.34^a	5.91^{a,b}	5.87^b	0.232	0.034	0.680	0.488
Simpson*	0.96	0.96	0.96	0.96	0.96	0.96	0.008	0.856	0.667	0.722

SBM, soybean meal; aSBM, autoclaved soybean meal; W, wheat; dHB, digestible hull-less barley; fHB, fermentable hull-less barley; G, grain; P, protein.

^{a,b}No G × P interaction, main effect of grain means separation shown. Means in a row with superscripts without a common letter differ, P < 0.05.

Table 5.10 Colonic short chain fatty acid concentration (umol/g wet weight) in pigs fed cereal grain based diets with autoclaved or untreated soybean meal

	SBM			aSBM				P values		
Items	W	dHB	fHB	W	dHB	fHB	SEM	Grain	Protein	G × P
Acetate	59.4	65.2	67.5	70.1	65.5	52.9	6.88	0.623	0.805	0.109
Propionate	30.6^z	$36.9^{y,z}$	49.8^y	33.6^z	$40.8^{y,z}$	34.6^z	3.25	0.013	0.304	0.009
Isobutyrate	1.10	1.17	0.91	1.15	1.11	0.85	0.165	0.156	0.843	0.909
Butyrate	11.3^z	$16.0^{y,z}$	19.8^y	$15.4^{y,z}$	$17.2^{y,z}$	12.7^z	2.35	0.294	0.732	0.049
Isovalerate	1.53	2.26	1.28	2.15	2.02	1.48	0.430	0.169	0.553	0.555
Total SCFA	105.7^z	$124.8^{y,z}$	149.1^y	$123.9^{y,z}$	$130.6^{y,z}$	107.4^z	10.8	0.289	0.449	0.009

SBM, soybean meal; aSBM, autoclaved soybean meal; W, wheat; dHB, digestible hull-less barley; fHB, fermentable hull-less barley; G, grain; P, protein.

[y, z]Main effects of G × P interaction shown. Means in a row with superscripts without a common letter differ, $P < 0.05$.
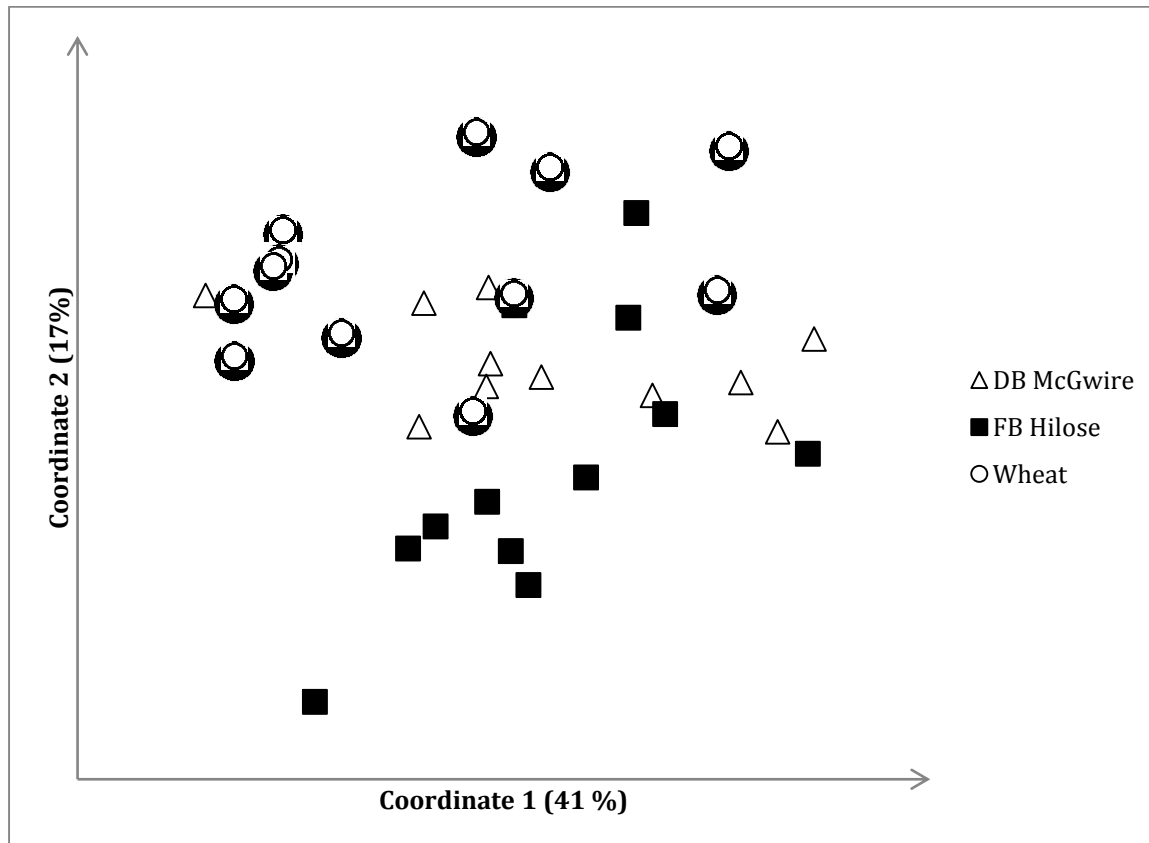
Figure 5.1 Principal Coordinate plot of weighted UniFrac distance metric based bacterial 16S rRNA sequence abundance in individual pig faeces samples. Pigs fed the FHB diet were clustered distinct from pigs fed DHB and W regardless of protein source.
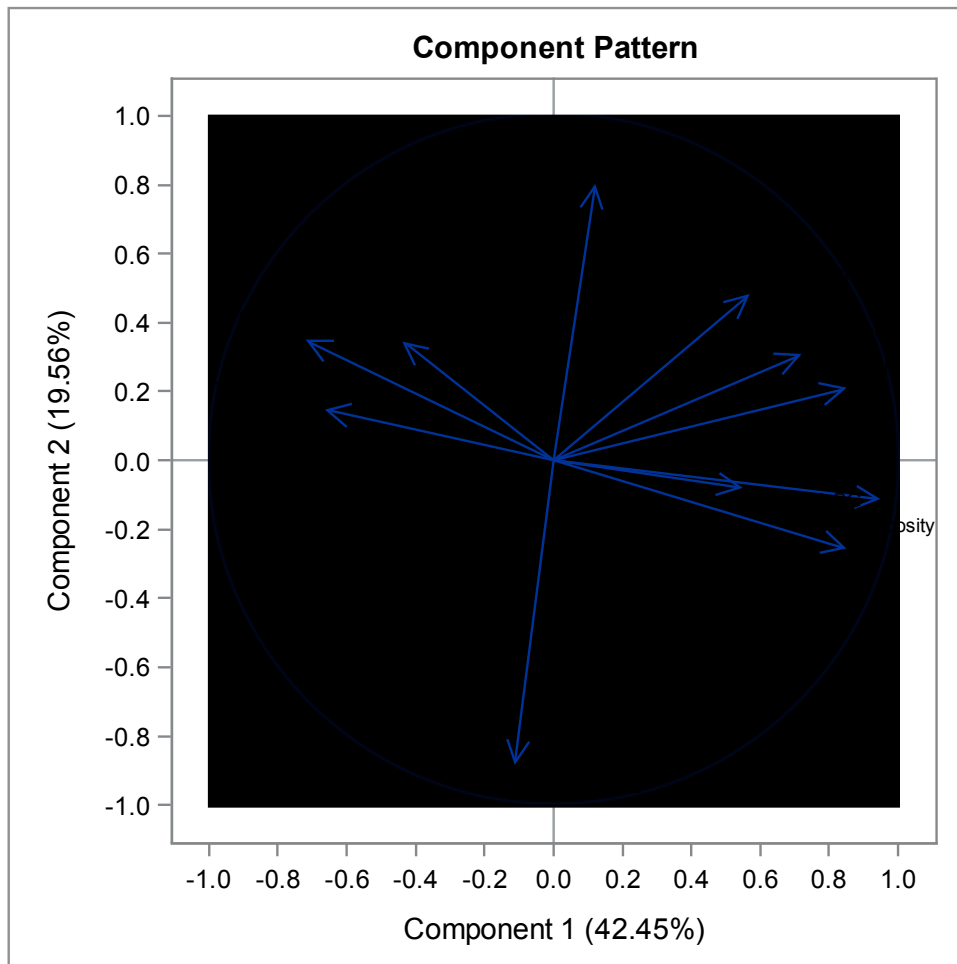
Figure 5.2 PCA loading plot of primary fecal bacterial phyla and components of cereal grain of interest in 36 pigs consuming cereal grain based diets with autoclaved or untreated soybean meal. The loading plot indicates relationships among observations and variables, respectively.

Table 5.11 Gastrointestinal histology in weaned pigs fed whole grain based diets with autoclaved or untreated soybean meal

	SBM			aSBM				P values		
Item	W	dHB	fHB	W	dHB	fHB	SEM	Grain	Protein	G × P
Crypt depth, *μm*										
Duodenum	301	301	344	355	307	313	23.7	0.348	0.534	0.097
Jejunum	258	251	260	274	257	249	21.8	0.561	0.721	0.561
Ileum	188	238	201	198	198	222	17.7	0.351	0.821	0.192
Villi height, *μm*										
Duodenum	367	332	348	339	341	352	17.6	0.628	0.730	0.526
Jejunum	378	330	359	337	373	376	32.4	0.860	0.800	0.341
Ileum	360	344	306	325	303	327	23.8	0.527	0.352	0.351

SBM, soybean meal; aSBM, autoclaved soybean meal; W, wheat; dHB, digestible hull-less barley; fHB, fermentable hull-less barley; G, grain; P, protein.

[a, b]No G × P interaction, main effect of grain means separation shown. Means in a row with superscripts without a common letter differ, $P < 0.05$.

Chapter 6. General Discussion

6.1 Summary and conclusions

Carbohydrates are a major constituent of human and swine diets. Among these, starch generally provides the most energy within a diet. Starch high in amylose is one of the resistant starch (RS) sources. The RS are the part of starch escaping small intestinal degradation and absorption thereby providing the hindgut microbiota a fermentative substrate. Increasing dietary RS may improve the gastrointestinal tract (GIT) micro-environment, by shifting microbial composition, increasing short chain fatty acid (SCFA) production, and reducing GIT pH (Bird et al., 2007; Bird et al., 2009; Regmi et al., 2011). However, fermentation of RS will increase heat loss through fermentation, making it less energy efficient than starch digestion that produces glucose that can directly be converted into ATP. We hypothesized that increasing RS by increasing dietary amylose would shift weaned pigs from starch digesters into starch fermenters, and thereby increase hindgut fermentation of starch and dry matter (DM) and alter the microbial and metabolite profiles, but also reduce growth. Chapter 2 data revealed that increasing dietary amylose to 63% vs. 0, 20, and 28% decreased ileal digestibility of DM and starch, increased hindgut fermentation, and increased cecal and colonic total SCFA and *Bifidobacterium* spp. However, pigs consuming 63% dietary amylose had lower feed intake and growth. Therefore, an optimal dietary amylose content to manipulate the GIT micro-environment without reducing growth may exist.

Characteristically, monogastric livestock receive the majority of dietary starch from whole grains, not purified sources. Whole grains are a complex matrix of macronutrients and can have a great degree of variation in composition of fermentable

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components. Increasing dietary fiber can reduce apparent ileal digestibility (AID) of nutrients and energy (Montagne et al., 2003; Högberg and Lindberg, 2004; Metzler-Zebeli et al., 2013). However, the association between naturally-occurring fermentable components, i. e., amylose, β-glucan, and total dietary fiber (TDF), within a cereal grain matrix and the site of energy and nutrient digestibility and resultant changes in grain net energy (NE) value are poorly understood. In Chapter 3, site of energy digestion in pigs moved from the small intestine to the hindgut and reduced standardized ileal digestibility (SID) of AA when whole grains are high in amylose, β-glucans, or TDF. Furthermore, increased fermentable fiber and digestible protein located within whole grains affected their NE value. Consequently, predicted NE value of grains high in fermentable fiber did not align with measured NE values, indicating that predictive equations may underestimate energy lost from fermentation. We conditionally accept that high levels of fermentable fiber may decrease efficiency of energy utilization, but that energy utilization is complex and governed by multiple mechanisms.

Dietary fiber is proposed to be required for proper development of the GIT (Montagne et al., 2003). Functional properties of fiber relate to microbial colonization, aiding in immune development, nutrient utilization, and pathogen exclusion (Slavin, 2010, 2013). Although dietary fiber positively manipulate the GIT environment, many studies have looked solely at specific and novel fiber and starch fractions, ignoring whole grain sources, until recently (Martinez et al., 2013). We hypothesized that whole grains, high in β-glucans, amylose, or TDF will increase post-ileal nutrient flow, increase hindgut substrate availability, and shift microbial profiles. In Chapter 4, consumption of barley, high in β-glucans or amylose, increased post-ileal starch and DM flow, increasing

fecal abundance of the Firmicutes phylum, including the genera *Dialster* and *Sharpea.*
Using principle component analysis, a relationship between ileal starch flow and fecal
abundance of specific phyla was established. Interestingly, hulled barley (LFB) with
moderate β-glucans and high ADF clustered away from wheat and hull-less barley
cultivars according to the weighted Unifrac distance metric. The greater content and
complex nature of ADF (lignin and cellulose) within hulled barley was likely the driving
force in changing the microbial community structure. Furthermore, increased lignin and
cellulose combined with β-glucans within the hulled barley likely created a distinct fiber
matrix, allowing for increased diversity. The tendency for wheat to alter microbial
composition more similar to hulled barley than hull-less barley cultivars is likely
attributable to a similar ADF composition.

When formulating diets for weaned pigs, nutritionists typically do not consider
how indigestible or fermentable nutrients will affect health or growth performance, but
rather consider digestible nutrient content in feedstuffs. Young pigs are typically fed
diets with a high crude protein content to partly meet their amino acid requirements.
However, proteolytic bacteria can ferment excess undigested protein and thereby
produce pro-inflammatory putrefactive metabolites (Macfarlane et al., 1986). Addition of
fermentable carbohydrates mitigates some negative effects of protein fermentation,
providing an alternative fermentative substrate (Jha and Leterme, 2012; Pieper et al.,
2012). However, using whole grains as fermentable fiber source has been mostly
overlooked. We hypothesized that feeding barley high in β-glucan or amylose vs. wheat
would increase growth performance, increase post-ileal nutrient flow, and shift microbial
and metabolite profiles regardless of protein source fed (heat damaged, autoclaved SBM

vs. SBM). In a 2 (autoclaved SBM vs. SBM) × 3 (high amylose or β-glucan barley vs. wheat) factorial arrangement, inclusion of autoclaved SBM decreased diet palatability thereby reducing intake and gain. However, consumption of barley high in amylose or β-glucans vs. wheat slowed nutrient flow through the hindgut, increased feed efficiency and DM digestibility, regardless of protein source (normal vs. autoclaved SBM). The microbiota shifted prominently with consumption of high β-glucan barley vs. wheat, increasing abundance of Firmicutes in colonic digesta. Chapter 5 revealed that cereal grains high in fermentable fiber may have beneficial properties that increase growth performance when fed to weaned pigs. Moreover, type of hindgut substrate availability seems to greatly influence gut microbial community structure. When comparing Chapter 4 and 5 results, we observed distinct similarities, within cereal grain cultivar, to shifts in microbial composition. Of particular interest is how wheat in Chapter 5 increased colonic digesta and fecal microbial diversity and tended to reduce colonic Firmicutes and increase Bacteroidetes, similar to observations for hulled barley in Chapter 4. This observation may be due to similarity in cellulose and lignin composition between wheat and hulled barley. However, substantial differences in diet composition and pig age between Chapter 4 and 5 does not allow for data compilation and further statistical comparison.

6.2 Limitations

Overall, the research conducted provided evidence on how starch composition whether from a purified source or within a cereal grain matrix can alter site, rate, and extent of digestion that subsequently affects microbial and fermentation profiles.

However, some challenges did exist throughout the project. In the first study (Chapter 2), the slaughter technique was used to collect digesta at multiple sites; however, we were unable to sample the same site multiple times to elucidate the kinetics of digestion (Bach Knudsen et al., 2006). Although not yet widely utilized in young pigs, the use of cannulas may be a better method to depict dynamics of nutrient flow over time. In Chapter 2, we utilized qPCR to quantify changes in a specific subset of microbiota. However, the GIT microbiome is a vast and dynamic system and its functions cannot be defined fully by a handful of microbiota. Therefore, use of qPCR limited the ability to quantitatively and qualitatively observe diet-induced microbial changes on a whole community scale.

For the study in Chapter 3 and 4, the cannulation method was utilized allowing digesta to be sampled multiple times to measure nutrient flow, digestibility, and microbial and metabolite profiles. To ensure dietary protein met or exceeded nutrient requirements a complex 'protein mix' was formulated and added to each diet. Using 2 reference diets containing energy and protein sources in the same ratio as test diets, we were able to calculate ingredient digestibility using the difference method. However, using complex diets and calculations to determine ingredient digestibility does not take into account interactions that may occur among feed ingredients and may introduce error. Inclusion of cellulose, an insoluble fiber, in reference diets may not have appropriately represented the fiber content of experimental barley and wheat based diets, changing digesta transit time and endogenous losses. Post-experimental analysis indicated that our cereal grains were above average in protein quantity and could have been used alone to meet nutrient requirements of growing pigs. Thus, sole use of cereal grains may have

decreased confounding effects of added protein. The study in Chapters 3 and 4 utilized a 6×7 Youden square design causing a lengthy study during which pigs dramatically gained weight between start and end of the study. The large weight range likely increased variation in observations and made accurate predictions of fasting heat production (FHP) difficult, a measurement needed to calculate the NE value of feedstuffs. In our study, we used metabolic body weight to estimate FHP (Moehn et al., 2013). However in the future, FHP should be measured by indirect calorimetry for more accurate quantification. Changes in animal weight directly changes feed intake that also affects FHP (de Lange et al., 2006; Labussière et al., 2010) and may have caused the high variations in measured NE of cereal grains. Moreover, activity related heat production was not measured and may have contributed to differences in heat production and NE value of feedstuffs, as activity related heat production changes between digestible starch and RS intake (Gerrits et al., 2012).

In chapter 5, we created a heat-damaged protein (autoclaved SBM) using the established variables (Pieper et al., 2012) by autoclaving SBM for 20 min at 121°C. However, protein fermentation was not observed, because changes in colonic branched chain fatty acids, i. e., isobutryate and isovalerate, were not detected. Previously, changes in branched chain fatty acid concentrations were used as qualitative indicators of protein fermentation (Jha and Leterme, 2012). However, to conclude whether or not excess protein fermentation occurred or could have occurred is impossible, because all cereal grains fed could have provided adequate fermentable carbohydrates mitigating any protein fermentation. Inclusion of a positive control to confirm protein fermentation would have added strength to the study design. Although perturbations in GIT function

such as diarrhea were not observed, implementation of a fecal scoring system would have strengthened this argument.

The intestinal microbiota interacts with the host thereby influencing nutritional, physiological, and immunological factors (Zoetendal et al., 2004). Characterizing microbial populations using specific regions of the 16S rRNA gene via sequencing can introduce error through primer bias. It is well known that the primers for regions V1-V3 used in the current study can under represent the Actinobacteria phyla. Use of whole genome shotgun sequencing can eliminate primer bias for specific hyper variable regions. The distribution of the microbiota is spatially distinct in the lumen of the GIT from mouth to anus and within the mucosa. Characterization of luminal microbiota in Chapter 4 and 5 limited our evaluation to the influence of microbes on nutritional factors; however, mucosa-associated microbiota likely have more influence on host physiological and immunological factors. To gain insight on how the microbiota interacts with nutritional, physiological, and immunological functions, distribution along the entire length of the GIT and within the mucosa needs to be characterized comprehensively.

6.3 Future research

In monogastric livestock production systems, starch is regarded as 100% digestible and assumed to provide the same amount of energy regardless of composition. Use of calorimetry in humans and rats indicate that the energetic value of RS is lower than digestible starch but measurements are inconsistent and variable (Tagliabue et al., 1995, Ranhotra et al., 1996). Introduction of a novel calorimetry method (Gerrits et al., 2012) utilizes differences in natural ^{13}C enrichment in feedstuffs to distinguish between

efficiency of energy utilization of digested vs. fermented starch. When evaluating nutritional and feeding properties of purified starch and starch sources, adoption of this novel calorimetry technique may accurately quantify the energy value of starch and starch sources. For humans and monogastrics animals, quantitatively defining the energy value of starch is important for weight maintenance or to predict growth performance.

The GIT microbiota is an important indicator and determinant of human and animal health with perturbations associated with various disease states. Thus, identification of the underlying mechanisms altering the microbiota is at the forefront of research. A vast number of studies have successfully altered the microbial and metabolite profiles by including prebiotic fiber into diets. However, limited information exists about physiological changes that occur when starch or fiber comes from its original matrix, as with whole grains. Past research has focused primarily on compositional changes of the microbiota; however, many functional redundancies exist between members. Thus, it may be more important to understand which microbial genes in the entire microbiome vs. which members of the microbiota are changing with dietary interventions. The concept of a "discrete structure" has been proposed (Hamaker and Tuncil, 2014), and is defined as "…the unique chemical structure, often within a fiber molecule, which aligns with encoded gene clusters in bacterial genomes" (Hamaker and Tuncil, 2014). Studying the "discrete structures" of fiber sources and which microbial genes are changing will help researchers to predict how starch, fiber, and whole grains change the structure and, more importantly, function of the microbiota.

Swine production is under scrutiny to reduce the use growth-promoting antibiotics while continuously increase production efficiency (WHO, 2000). Feeding pigs post-

weaning without antibiotics represents a challenge due to the high incidence of post-weaning diarrhea and growth lags that are associated with decreases in the loosely defined 'gut health' (Lallès et al., 2007). However, researchers have not quantified the mechanisms of growth-promoting antibiotics, so feeding strategies to mitigate problems associated with post-weaning diarrhea have only been partially successful. Therefore, the mechanisms of growth-promoting antibiotics on 'gut health' need to be defined quantitatively, including for optimal gut architecture and function, digestive efficiency, immune function, and gut microbiology.

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