MODULATION OF INTESTINAL CELL DIFFERENTIATION BY DIFFERENT FIBER SOURCES AND EVALUATION OF USING A MODIFIED THREE-STEP PROCEDURE TO PREDICT DIGESTIBLE AND METABOLIZABLE ENERGY CONTENT IN GROWING PIGS

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Dedication

I dedicate this thesis to my professors, who taught me to think, understand, share, and express; and to my family and friends, who expressed their support, love, and care.

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Abbreviations

ADF	Acid detergent fiber
ADFI	Average daily feed intake
ADG	Average daily gain
ATOH1	Atonal bHLH transcription factor 1
ATTD	Apparent total tract digestibility
CBC	Crypt base columnar
CCSDS	Computer-controlled simulated digestion system
CHGA	Chromogranin A
DDGS	Corn distillers dried grains with solubles
DLL1	Delta like 1
DLL4	Delta like 4
FABP	Fatty acid binding protein
GIT	Gastrointestinal tract
HES1	Hairy and enhancer of split 1
IDF	Insoluble dietary fiber
IVDMD	*In vitro* dry matter digestibility
$IVDMD_f$	*In vitro* dry matter digestibility from simulated large intestine fermentation
$IVDMD_h$	*In vitro* dry matter digestibility from simulated gastric and small intestinal hydrolysis
$IVDMD_{tn}$	*In vitro* dry matter digestibility from simulated total tract digestion

LGR5	Leucine-rich repeat-containing G protein-coupled receptor 5-expression
LYZ	Lysozyme
M cells	Membranous or microfold cells
MUC	Mucin
NDF	Neutral detergent fiber
NDSC	Neutral detergent soluble carbohydrate
NFC	Non-fiber carbohydrate
NIRS	Near infrared reflectance spectroscopy
NSC	Nonstructural carbohydrate
NSP	Nonstarch polysaccharides
OLFM4	Olfactomedin 4
SBH	Soybean hulls
SCFA	Short chain fatty acid
SDF	Soluble dietary fiber
TA	Transit amplifying
TDF	Total dietary fiber
VFA	Volatile fatty acids
WDGS	Wet distillers grains with solubles
WNT3a	Wingless-type MMTV integration site family 3A
WS	Wheat straw

Chapter 1. Introduction and literature review

INTRODUCTION

Growing populations of people around the world have led to increased demand for meat, milk, and eggs. Current projections are that agricultural productivity will need to increase substantially to feed about 9.6 billion people by 2050 (FAO, 2015). The grains consumed by livestock represent 41% of the total grain production, and the world will require more cereal grains to meet food and feed needs by 2050 (IAASTD, 2009). Pork is the most widely consumed meat in the world, and swine feed represents 26% of total feed consumed by livestock, poultry, and in aquaculture (Alltech, 2016). Therefore, to meet the increasing need for grains for human consumption, as well as meeting the increased needs for feed ingredients in animal production, by-products from various agricultural and industrial processes, including grain processing, must be used to a greater extent in swine diets. In addition, technologies need to be developed, evaluated, and implemented to increase the efficiency of using energy and nutrients from existing feed ingredients (Woyengo et al., 2014). Several technologies have been developed for increasing caloric and nutrient utilization efficiency including reducing ingredient and diet particle size (Liu et al., 2013), dietary supplementation with exogenous enzymes like proteases, carbohydrases, and phytase (Zijlstra et al., 2010), formulating diets based on amino acid standardized ileal digestibility coefficients (Landero et al., 2011), and removing antinutritional factors by dehulling (House et al., 2003), air classification (Zhou et al., 2013), and heat treatment (Jezierny et al., 2010).

Historically, the use of various by-products in swine feeds in the U.S. has been limited due to the abundance and relatively low prices of corn and soybean meal, which

supply the majority of energy and amino acids to swine diets. However, the increased use of corn (133 million MT) for ethanol production (RFA, 2016) has resulted in increased availability of corn distillers dried grains with solubles (DDGS) as a partial replacement for corn and soybean meal in commercial swine diets. Soybean hulls (SBH) produced from soybean meal processing and wheat middlings produced from wheat flour processing are also available in large quantities in the U.S. (Stewart et al., 2013). However, these abundant by-products contain substantially more fiber content than corn and soybean meal, which is not well utilized by monogastric animals.

High fiber by-products are becoming major components in swine diets due to their competitive prices ($/tonne, Table 1.1), availability of significant quantities (Stein and Shurson, 2009), potential gut health benefits for growing pigs (Whitney et al. 2006), and animal welfare (satiety) benefits in sow diets (Bindelle et al., 2008). However, there are challenges when adding these by-products to swine diets. One of the challenges is the high fiber content (generally considered as NDF > 18.7%; Sauvant et al., 2004), which depresses nutrient digestibility in swine (Dégen et al., 2007). In addition, increased use of high fiber ingredients has led to the need to use the net energy (NE) system instead of the metabolizable energy (ME) system when formulating swine diets because of the high heat increment and energy losses induced by greater dietary concentrations of fiber (NRC, 2012). Considering the cost per Mcal of NE, SBH are less costly than soybean meal, but more costly than corn, wheat, corn DDGS and wheat middlings; the price of corn DDGS is comparable with corn price; and wheat middlings is less costly than wheat (Table 1.1). Overall, these high fiber ingredients are less expensive or similar in price to their original grain from which they are derived. Another challenge is the substantial

variability in digestible energy and nutrient content among sources of various by-products, which reduces the accuracy of diet formulation when dynamic estimates of nutrient loading values for specific sources in feed manufacturing are not available. As a result, research is needed to better understand how to utilize high fiber ingredients efficiently in commercial swine diets. One of the essential areas of research is to understand the mechanism of how high fiber ingredients depress nutrient utilization. Another important area of research is to develop accurate, rapid, and relatively inexpensive methods to dynamically estimate digestible energy and nutrient content of these high fiber ingredients (Pork Checkoff Report, 2012). Therefore, the foci of this thesis are to: 1) investigate the mechanisms of how high fiber ingredients affect nutrient utilization in swine by understanding how dietary fiber affects gastrointestinal development, specifically cell proliferation and differentiation of the small intestine, and 2) to develop and evaluate a modified three-step *in vitro* method for rapid estimation of the digestible and metabolizable energy content and fiber digestibility and fermentability among high fiber feed ingredients.

Table 1.1 Comparison of feed ingredient price, NE content, and cost per Mcal of NE[1]

Ingredient	**Corn**	**Corn DDGS[2]**	**Soybean meal, dehulled, solvent extracted**	**Soybean hulls (SBH)**	**Wheat**	**Wheat middlings**
GE, kcal/kg	3933	4710	4256	4210	3788	3901
NE, kcal/kg	2672	2343	2087	989	2472	2113
NE: GE, %	68	50	49	23	65	54
Price, $/tonne	132.6	134.9	332.7	102.8	183.5	90.9
Cost $/Mcal NE	0.050	0.058	0.159	0.104	0.074	0.043

[1]Price of feed ingredients are referenced from ingredient market published in Feedstuffs (January, 2016 to August, 2016), GE and NE of ingredients referenced from NRC (2012) for corn, soybean meal, corn DDGS, SBH, and wheat middlings

[2]Distillers dried grains with solubles，6 < Oil < 9%

LITERATURE REVIEW

1.1 Definition of dietary fiber

The study of dietary fiber began in the scientific fields of food science and human nutrition. Hipsley (1953) was the first to suggest the term “dietary fiber” as a sum of lignin, cellulose, and hemicellulose, which were considered as unavailable carbohydrates (McCance and Lawrence, 1929; Table 1.2). Subsequently, Trowell (1972) defined dietary fiber as “the skeletal remains of plant cells that are resistant to digestion by enzymes of man”, which he later updated to “the residue derived from plant cell walls that is resistant to hydrolysis by human alimentary enzymes” (Trowell, 1976). Numerous reviews have been published that discuss the accuracy of various definitions of dietary fiber, which led to an agreement that the definition of dietary fiber should be based on both chemical and physiological properties (Lee and Prosky, 1995; AACC, 2001; Champ *et al.*, 2003), and was described as “edible parts or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine” (AACC, 2001). In more recent reviews, dietary fiber was separated between functional fiber and total fiber (Hellwig et al., 2006). Under this classification system, dietary fiber was defined as “the carbohydrate and lignin that are intrinsic and intact in plants and that are not digested and absorbed in the small intestine”. Functional fiber was defined as “isolated or purified carbohydrates that are not digested and absorbed in the small intestine and that confer beneficial physiological effects in human”, and total fiber is the sum of dietary fiber and functional fiber. In the most recent review, there are 4 clinically significant categories of fiber supplements that benefit human health: insoluble dietary fiber; soluble non-viscous and fermentable dietary fiber; soluble

viscous and readily fermented dietary fiber; and soluble viscous and non-fermented dietary fiber (McRorie and Fahey, 2015). This method of categorization considers the physiologic effect of dietary fiber based on its characterization (solubility, viscosity, and fermentability) and includes not only non-starch polysaccharides, lignin, cutin, suberin, waxes, and fibers found in animals, but also chemically synthesized carbohydrate compounds (McRorie and Fahey, 2015). This new system is the most comprehensive categorization of dietary fiber thus far.

Table 1.2 Summary of definitions of dietary fiber

Reference	Terminology
McCance and Lawrence (1929)	Unavailable carbohydrates
Hipsley (1953)	**Dietary fiber**: includes lignin, cellulose, and hemicellulose
Southgate (1969)	**Unavailable carbohydrates**: carbohydrates that are not hydrolyzed by any enzymes secreted into the human digestive tract
Trowell (1972)	**Dietary fiber**: the skeletal remains of plant cells that are resistant to digestion by enzymes of man
Spiller et al.(1975)	**Plant fiber**: 1) nonpurified plant fiber-plant cell wall in its natural state, containing whatever associated substances might be present 2) purified plant fiber-structural polymers have been isolated and purified
Spiller et al. (1976)	**Plantix** (plant and matrix, purified plant fiber, dietary fiber): sum of cellulose, hemicellulose, mucilages, pectins, gums, and lignin; or a single entity of one of them **Complantix** (complex plantix): plantix + associated plant cell wall factors (waxes, cutins; cell wall-bound undigestible proteins; other cell wall-bound undigestible sustances)
Trowell (1976)	**Dietary fiber**: the residue derived from plant cell walls that is resistant to hydrolysis by human alimentary enzymes **Dietary fiber complex**: all of the structural polymers of dietary fiber together with all associated chemical substances naturally associated with, and concentrated around, the structural polymers, especially if these substances are considerably reduced by modern food processing
Southgate (1982)	**Dietary fiber**: sum of lignin and the non-α-glucan-polysaccharides in food or diet
van Soest et al. (1991)	**Dietary fiber**: includes lignin and all polysaccharides resistant to mammalian digestive enzymes
Asp (1995)	**Dietary fiber**: indigestible material as measured with a standard method, such as an enzymatic, gravimetric AOAC[1] method, and with addition, when relevant, of carbohydrates fulfilling the following criteria: 1) indigestible in the human small intestine; 2) one or several physiological effects typical for dietary fiber; 3) measureable in the food in question with a reasonably simple method
Lee and Prosky (1995)	**Dietary fiber**: oligo- and polysaccharides and lignin that are resistant to hydrolysis by human alimentary enzymes
AACC[2] (2001)	**Dietary fiber**: the edible parts or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with

	complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation.
Hellwig et al. (2006)	**Dietary fiber**: the carbohydrates and lignin that are intrinsic and intact in plants and that are not digested and absorbed in the small intestine **Functional fiber**: consists of isolated or purified carbohydrates that are not digested and absorbed in the small intestine and that confer beneficial physiological effects in humans **Total fiber**: the sum of dietary fiber and functional fiber
McRorie and Fahey (2015)	**Dietary fiber**: with the following category based on dietary fiber characterization: insoluble dietary fiber; soluble non-viscous and fermentable dietary fiber; soluble viscous and readily fermented dietary fiber; soluble viscous and non-fermented dietary fiber. It includes all non-starch polysaccharides, non-digestible oligosaccharides, other fibers found in the plants, resistant starch, and chemical synthesized carbohydrate compounds

[1]AOAC: American Officials of Analytical Chemistry.

[2]AACC: American Association of Cereal Chemistry.

1. 2 Classification of carbohydrate

Carbohydrates may be classified according to degree of polymerization, composition of constituent sugars, and types of glycosidic linkages present (Englyst and Hudson, 1996). Based on sugar composition, carbohydrates in feed include: monosaccharides, disaccharides, oligosaccharides, and polysaccharides (NRC, 2012). Partitioning dietary carbohydrates has been characterized based on current analytical methods and nutritional or physiologic functions relative to animal digestive function (Figure 1.1; NRC, 2007). These categories may not include all carbohydrates produced by plants, and some non-carbohydrate components are included because they are components of the specific analytical fractions, such as organic acids, lignin, and phenolics (NRC, 2007). Specific fructans can be categorized as either fructooligosaccharides or fructan polysaccharides depending on degree of polymerization (van den Ende et al., 2013). Total starch may be comprised of starch present in a chemical form that is easily digested to glucose, or it can be resistant to enzymatic

hydrolysis. As a result, some resistant starch may appear in other fiber or carbohydrate fractions (Method 2002.12; AOAC, 2012). Furthermore, some hemicellulose may be soluble in neutral detergent, and thus recovered in the non-fiber carbohydrate (NFC)/neutral detergent soluble carbohydrate (NDSC) fraction (Mertens, 2003). Crude fiber analysis based on chemical digestion and the amount of cell wall constituents varies by feed ingredient, and therefore, does not fit the nutritional definition of dietary fiber (Mertens, 2003). From a nutritional perspective, nonstarch polysaccharides (NSP) include all polysaccharides except starch. However, the analytical method for NSP may recover a variable amount of fructan polysaccharide (NRC, 2007) and therefore the prebiotic function of fructan is not accurately estimated (Kolida and Gibson, 2007). Measurement of total dietary fiber (TDF) includes all carbohydrates resistant to mammalian digestion, and represents both soluble and insoluble carbohydrate fractions. However, the analytical method for TDF and soluble dietary fiber (SDF) does not recover oligosaccharides, and may recover a variable amount of fructan polysaccharides (McCleary et al., 2012). Therefore, the analytical method chosen to measure various fiber fractions can provide incomplete and misleading information relative to the actual nutritional and physiological properties of dietary fiber, and indicates that better methods are needed.

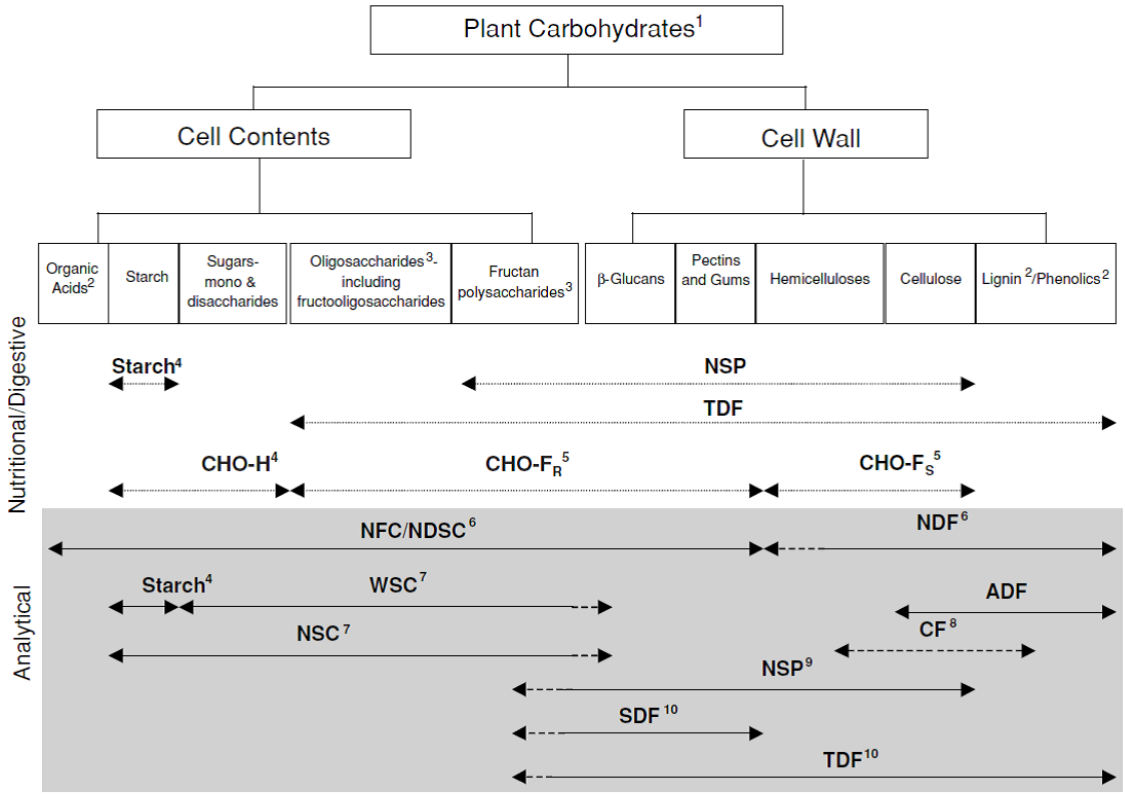

Figure 1.1 Fractionation of plant carbohydrates and related compounds (NRC, 2007)

CF = crude fiber; CHO-H hydrolysable carbohydrates, CHO-F_S: slowly fermentable carbohydrates, CHO-F_R: rapidly fermentable carbohydrates, NDSC: neutral detergent soluble carbohydrates, NFC: nonfiber carbohydrates; NSC: nonstructural carbohydrates; NSP: nonstarch polysaccharides; SDF: soluble dietary fiber; TDF: total dietary fiber; WSC: water-soluble carbohydrates

[1]Major categories of carbohydrates and associated substances are showed. These categories may not include all carbohydrates produced by plants.

[2]Some noncarbohydrate components are included here as they are components of the specific analytical fractions.

[3]Specific fructans can be catergorzed as either fructooligosaccharides or fructan polysaccharides depending on degree of polymerization.

[4]A variable fration of total starch can be resistant to enzymatic hydrolysis and thus some starch may appear in other nutriental fractons.

[5]Fermentability of gums may be variable.

[6]Some hemicellulose may be soluble in neutral detergent and thus recovered in the NFC/NDSC fraction, rather than the NDF fraction.

[7]Recovery of compounds in the analytical WSC fraction (and thus the NSC fraction when NSC is approximated as starch + WSC) may depende on methodology used.

[8]Amount of cell wall constituents included in CF analysis varies by feed.

[9]From a nutritional perspective, NSP includes all polysaccharides except starch. However, the analytical method for NSP may recover a variable amount of fructan polysaccharide.

[10]From a nutritional prespective, TDF includes all carbohydrates resistant to mammalian digestion. However, the analytical method for TDF (and SDF) does not cover oligosaccharides and may recover a variable amount of fructan polysaccharides.

1.3 Analytical methods of dietary fiber

There are many analytical methods to chemically determine dietary fiber concentration in human food, animal feed, and feed ingredients (Table 1.3), but these analytical methods often overlap or may exclude fractions of other distinctly different carbohydrate fractions in a feedstuff (Mertens, 2003). All methods of measuring dietary fiber include two basic steps: 1) chemical digestion of the diet or feed ingredient, and 2) quantification of the undigested residue remaining (Urriola et al., 2013). The digestion of feeds or ingredients can be done by using chemicals (e.g., acid, alkali, and detergent) or enzymes (e.g., amylase, glucoamylase, and protease). The quantification of the undigested residues involves using a gravimetric method to weigh the residues, and gas-liquid chromatography or high-performance liquid chromatography to measure chemical compounds in the residues.

Crude fiber is determined using a chemical-gravimetric method, and is one of the components included in the proximate analysis of a feed or feed ingredient. The proximate analysis, or Weende system of feed analysis, was developed in Weende Experimental Station in Germany in 1851, and includes analysis of moisture, ash, crude protein, crude fat (ether extract), nitrogen-free extract (determined by difference by subtracting the concentrations of the other proximate components from 100), and crude fiber (NRC, 2007). Carbohydrates are separated into crude fiber and nitrogen-free extract using this method. The specific procedure of crude fiber analysis is described in AOAC 978.10 (Figure 1.2). The most significant problem with using this procedure, is that the soluble fiber, and part of the lignin, cellulose, and hemicellulose is found in the nitrogen-free extract, so the analyzed concentration of crude fiber does not adequately describe the

actual fiber composition of a feed ingredient (Hellwig et al., 2006; NRC, 2012). However, the Weende crude fiber procedure is still being used by regulatory agencies and in international feed ingredient trading because it is robust and repeatable.

Measurement of detergent fiber is also a chemical-gravimetric procedure (Figure 1.3) that was initially developed by van Soest (van Soest, 1963). This method separates non-starch polysaccharides into NDF, ADF, and lignin (Robertson and Horvath, 2001). The concentration of cellulose is calculated as the difference between the concentration of lignin and ADF, and the concentration of hemicellulose is calculated as the difference between ADF and NDF. When analyzing substrates that contain mainly insoluble fiber, this method is relatively accurate, fast, and reproducible.

Total dietary fiber consists of the remnants of edible plant cells, polysaccharides, lignin, and associated substances resistant to digestion by the alimentary mammalian digestive system (Trowell, 1985). Specifically, TDF includes oligosaccharides, polysaccharides, β-glucans, pectins and gums, hemicellulose, cellulose, lignin (NRC 2007). The TDF procedure (Figure 1.4) is a more comprehensive enzymatic-gravimetric method to quantify all of the fiber fractions in a feed ingredient, and also separates the various carbohydrates into soluble and insoluble fiber fractions (NRC 2012). Results obtained from using the TDF analytical procedure more closely represent the nutritional and physiological effects of the TDF fraction in a feed ingredient than results obtained with the detergent procedures (Mertens, 2003). The major challenge of using the TDF procedure is that results obtained are less reproducible than results obtained with the detergent procedure (NRC, 2012).

The methods used to determine NSP content of feed and feed ingredients include the Uppsala method (Figure 1.5) and the Englyst method. The Uppsala method calculates dietary fiber as the sum of amylase-resistant polysaccharides, uronic acids, and Klason lignin (AOAC, 2012). The residue is separated into soluble and insoluble fractions by using 80% ethanol. The neutral sugars released are quantified as alditol acetate derivatives and uronic acids by gas chromatographically (Jones and Albersheim, 1972). The Englyst method (Englyst and Hudson, 1987) is similar to the Uppsala method, but it excludes lignin and resistant starch from the final value. Both methods lack reproducibility and are more expensive than other methods.

In summary, there is no single method of analysis for dietary fiber that accurately measures all carbohydrates that represent the nutritional definition of dietary fiber (NRC, 2007). The TDF procedure is the method that captures the most carbohydrates that fit the definition of dietary fiber, while the NSP method can determine the concentrations of single neutral sugars, which is beneficial for understanding the mechanisms of how specific components of dietary fiber affect the nutritional and physiological responses of animals.

Table 1.3 Analytical methods of dietary fiber

Reference	Name	Method	Measures	Main concerns
AOAC[1] (2012)	Weende system; AOAC 978.10	Chemical-gravimetric	Crude fiber (cellulose, hemicellulose, and lignin)	The recovery of fiber components are not complete, no relationship with current dietary fiber definition
AOAC (2012)	AOAC 2002.04	Enzymatic/Chemical-gravimetric	Amylase-treated neutral detergent fiber (cellulose, hemicellulose, and lignin)	Soluble dietary fiber is not recovered
AOAC (2012)	AOAC 973.18	Chemical-gravimetric	Acid detergent fiber (cellulose and lignin) and lignin	
AOAC (2012)	AOAC 991.43	Enzymatic-gravimetric	Total, soluble, and insoluble dietary fiber	Quantify only a portion of resistant starch; inulin or polydextrose are not quantified
Englyst and Hudson (1987)	Englyst method	Enzymatic-chemical or GLC or HPLC	Nonstarch polysaccharides	Lack of reproducibility, expensive
AOAC (2012)	Uppsala method; AOAC 994.13	Enzymatic-chemical or GLC or HPLC	Neutral sugar residues, uronic acid residues, and Klason lignin	Lack of reproducibility, expensive
AOAC (2012)	AOAC 995.16	Enzymatic	β-Glucans	
AOAC (2013)	AOAC 997.08	Enzymatic and ion-exchange chromatography	Fructans	
AOAC (2012)	AOAC 2000.11	Ion chromotography	Sugars/Polydextrose	
AOAC (2012)	AOAC 2002.02	Enzymatic	Resistant starch	Consistent with *in vivo* data

[1]AOAC: American Officials Analytical Chemistry.

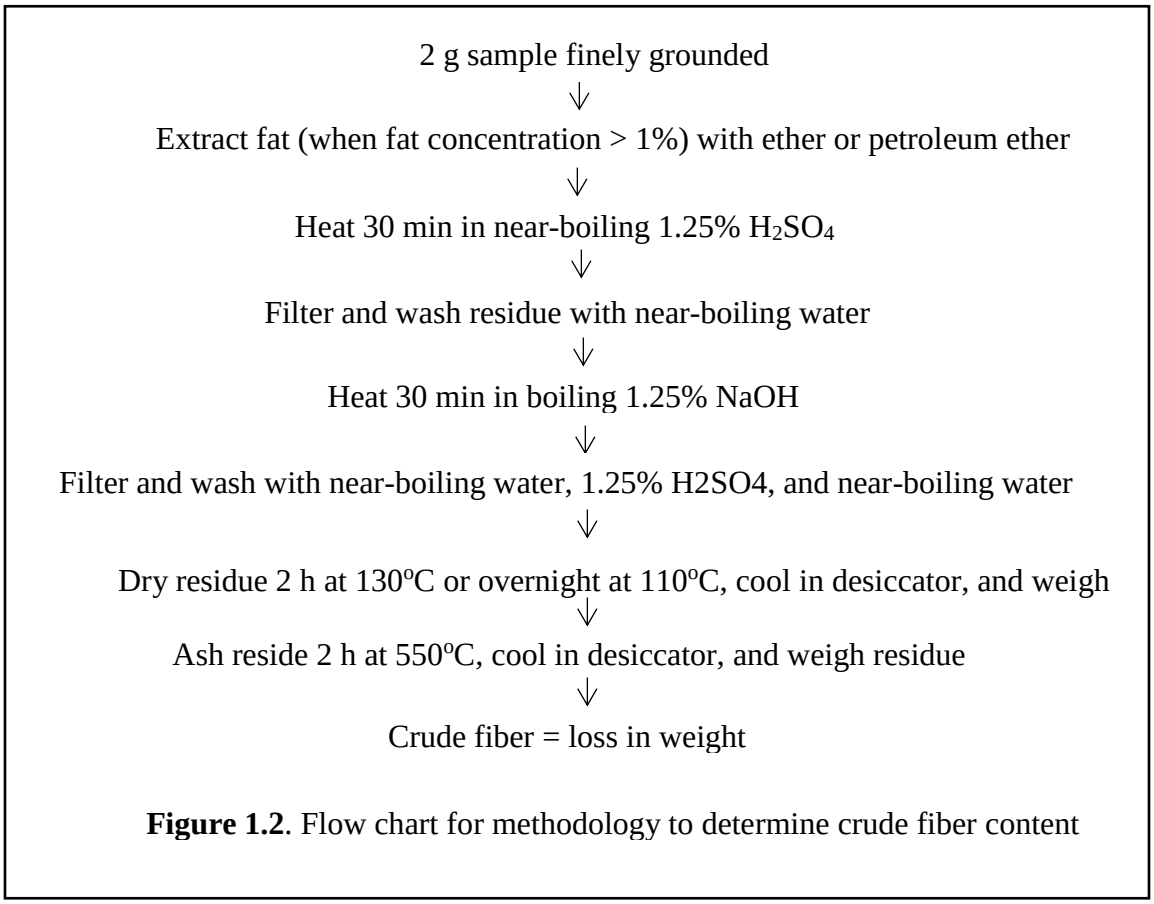

Figure 1.2. Flow chart for methodology to determine crude fiber content

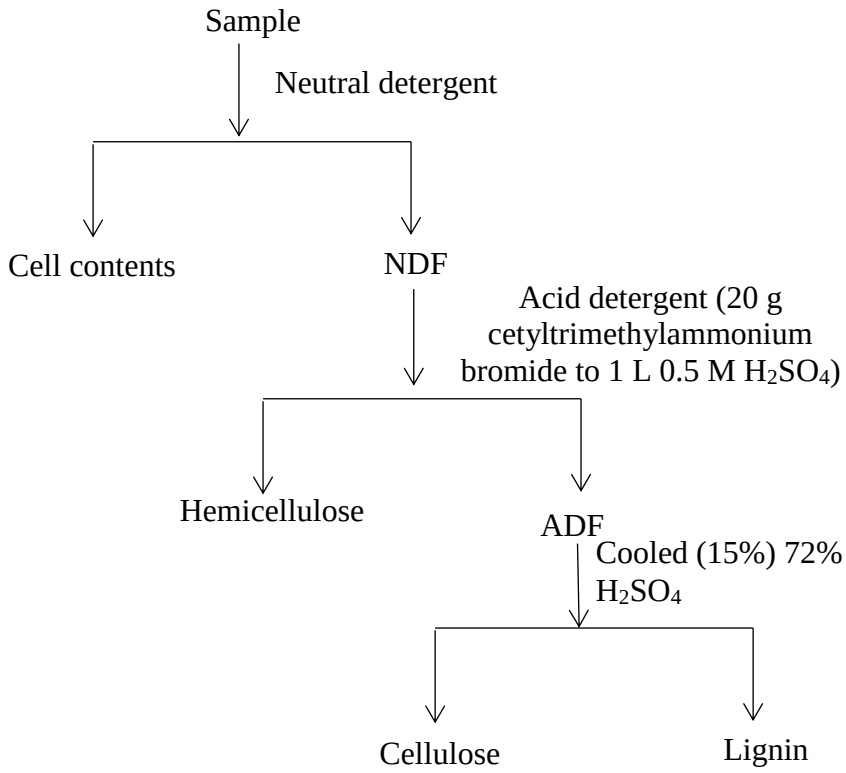

Figure 1.3 Flow chart of methodology for detergent fiber analysis system

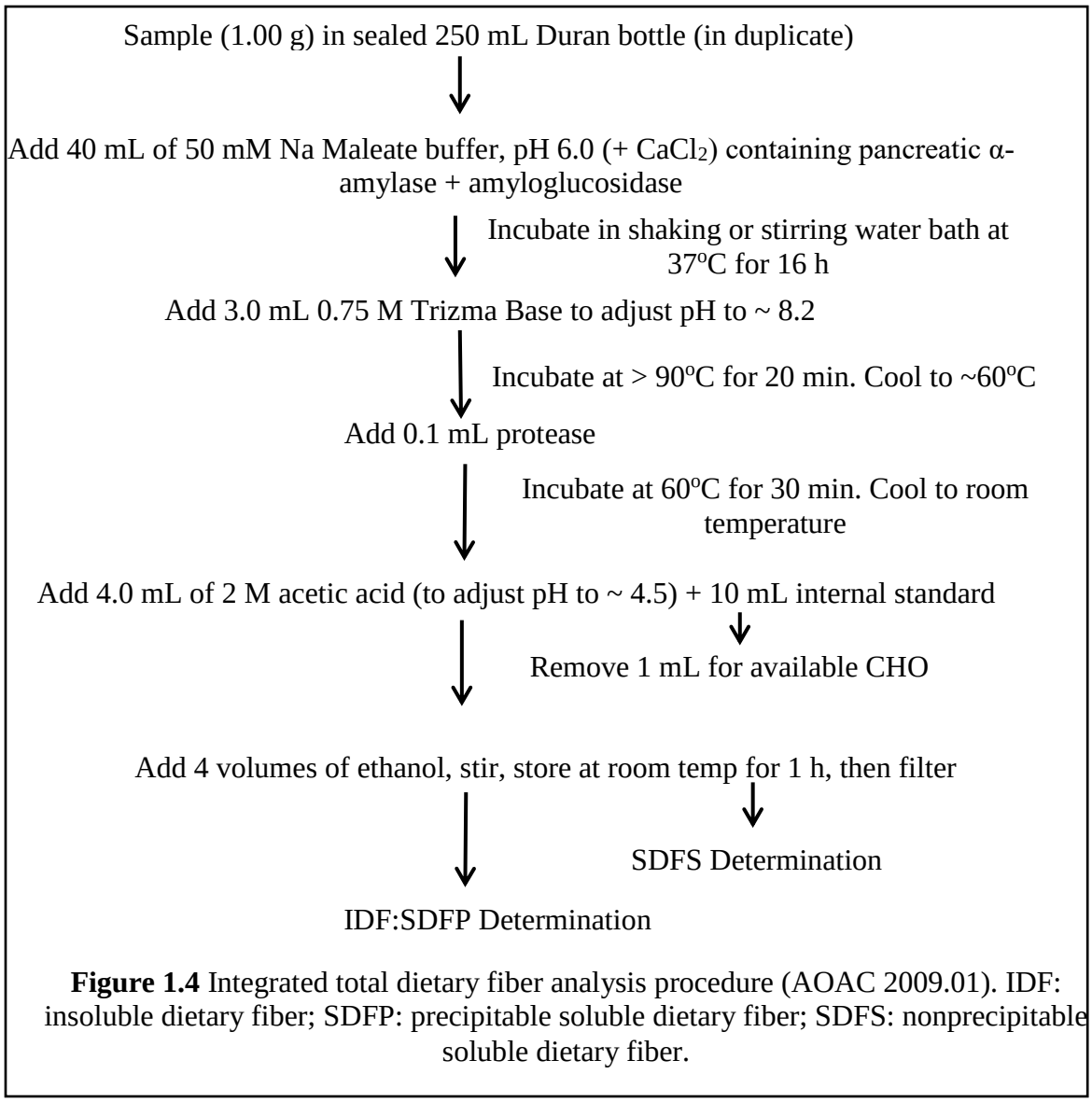

Figure 1.4 Integrated total dietary fiber analysis procedure (AOAC 2009.01). IDF: insoluble dietary fiber; SDFP: precipitable soluble dietary fiber; SDFS: nonprecipitable soluble dietary fiber.

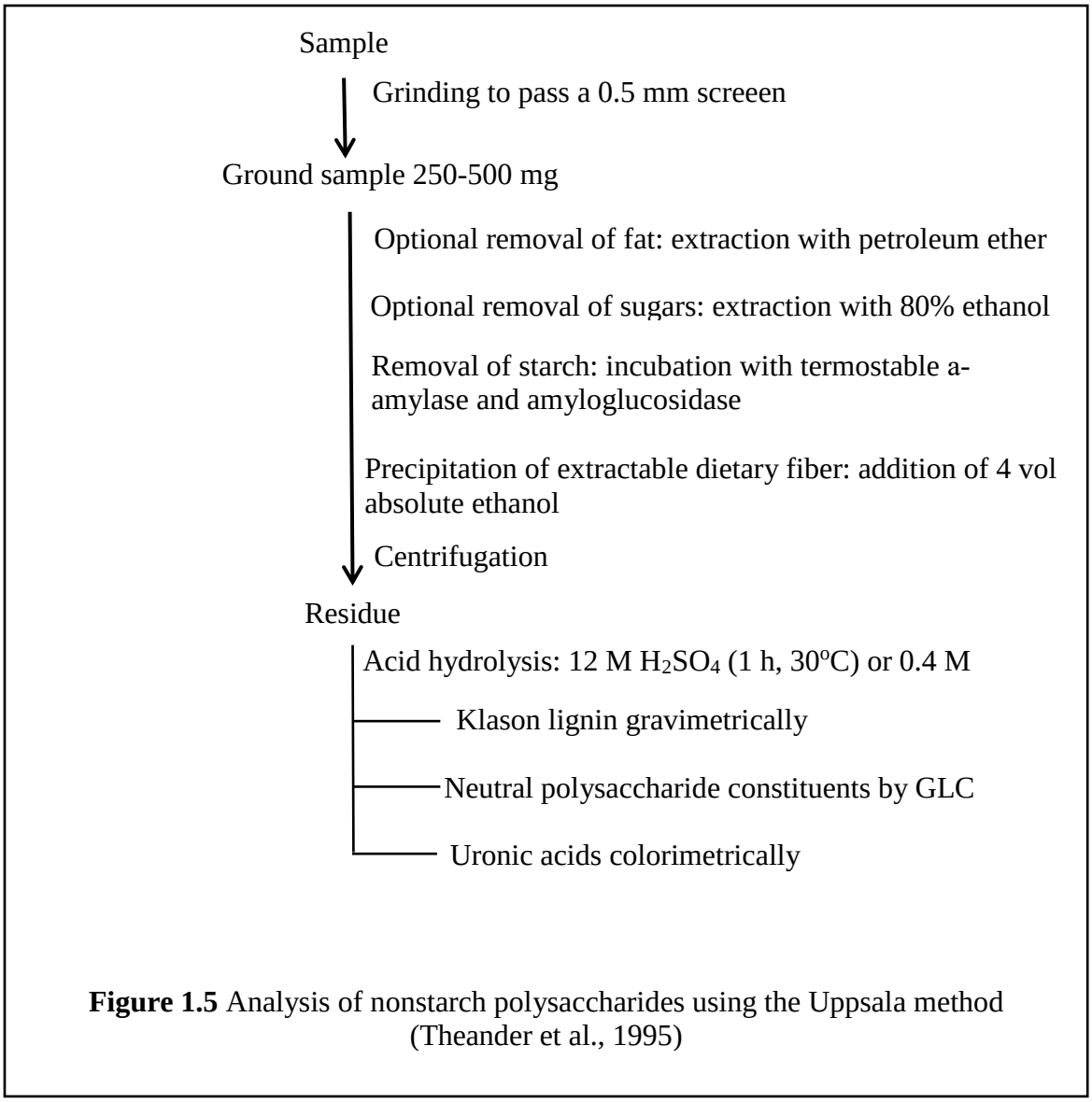

Figure 1.5 Analysis of nonstarch polysaccharides using the Uppsala method (Theander et al., 1995)

1.4 Physiological properties of dietary fiber

Dietary fiber is derived mainly from plant cell walls that consist of a several types of polysaccharides that are often associated with proteins and phenolic compounds, together with the phenolic polymer lignin (Theander et al., 1989). The major physiological properties of dietary fiber include water binding capacity, solubility, viscosity, and fermentability.

Water holding capacity and water binding capacity have been used interchangeably in the literature because both reflect the ability of a fiber source to incorporate water within its matrix (Bach Knudsen, 2001). Water binding capacity is determined by the physico-chemical structure of the molecules, and by the pH and electrolyte concentration of the surrounding fluid (Kay, 1982). Water holding capacity describes the quantity of water that can be found in fiber without the application of any external force (Robertson et al., 2001). Solubility of dietary fiber refers to the ability of dietary fiber to dissolve in water, dilute acid, dilute base, or a buffer or enzyme solution that mimics the enzyme solution existing in the gastrointestinal tract (GIT; Oakenfull et al., 1998). Solubility of dietary fiber depends on its molecular structure (Oakenfull et al., 1998). Regular and ordered structures like cellulose, or linear arabinoxylans do not bind with water very well, and usually have low solubility because their linear structure increases the strength of the non-covalent bonds that stabilize the ordered conformation (Thibault et al., 1992). Viscosity refers to the ability of dietary fiber to thicken or form gels in solution (Dikeman and Fahey, 2006). The viscosity is primarily dependent on the molecular weight and concentration of the polymer. Large molecules increase the viscosity of diluted solutions, and the extent of this occurance depends primarily on the

volume they occupy (Bach Knudsen, 2001). The fermentability of dietary fiber refers the chemical composition and the ability of microflora in the GIT to ferment and convert the fiber to volatile fatty acids (VFA). The susceptibility of dietary fiber to microbial fermentation varies depending on the accessibility of dietary fiber to the microbial population in the hindgut (Oakenfull, 2001).

These properties (water holding capacity, solubilty, viscosity, and fermentability) of dietary fiber interact with each other. Generally, dietary fiber with greater fermentability has greater viscosity, solubility, and water holding capacity, but insoluble dietary fiber (IDF) is usually not associated with viscosity (Takahashi et al., 2009). Each dietary fiber source has a unique combination of these properties, and there is no universal criteria to adequately characterize all of the physiological properties of dietary fiber. For example, some synthetic fiber components, such as carboxylmethylcellulose, has high viscosity but low fermentability (Smits et al., 1997). Another example is that fructans in inulin are soluble and fermentable, but non-viscous (Eswaran et al., 2013). Therefore, research investigating the physiological properties of dietary fiber must involve quantitatively understanding the chemical components of fiber in each ingredient being evaluated.

1.5 Fermentation of dietary fiber in swine

Dietary fiber cannot be hydrolyzed by gastric and small intestinal digestive enzymes, but dietary fiber can be fermented by the microflora in the large intestine. There are about $\log^{10\text{-}11}$ CFU/g of anaerobic bacteria in the large intestine of swine (Jensen and Jørgensen, 1994), which ferments dietary fiber and produces VFA, various gases (CO_2, H_2, and CH_4), and bacterial cell biomass (Figure 1.6; Bindelle et al., 2008). The main

components of VFA are acetate, propionate, and butyrate. Approximately 90% of the VFA produced are absorbed by colonocytes (Wong et al., 2006), and can be used as an energy source by other tissues in different ways (Bindelle et al., 2008). Briefly, acetate is transported to the liver and then acts as an energy substrate for muscle tissue. Propionate is converted to glucose in the liver. Butyrate is used primarily by the colonocytes, and provides a major source of energy for its metabolic activities (Rérat et al., 1987). The specific energy value from VFA is shown in Table 1.4.

Table 1.4 Energy value from VFA production (Blaxter, 1989; Christensen et al., 1999)

Item	ATP, mol/mol VFA	Energy, kcal/mol VFA
Acetate	10	209.6
Propionate	18	366.4
Butyrate	28	522.2

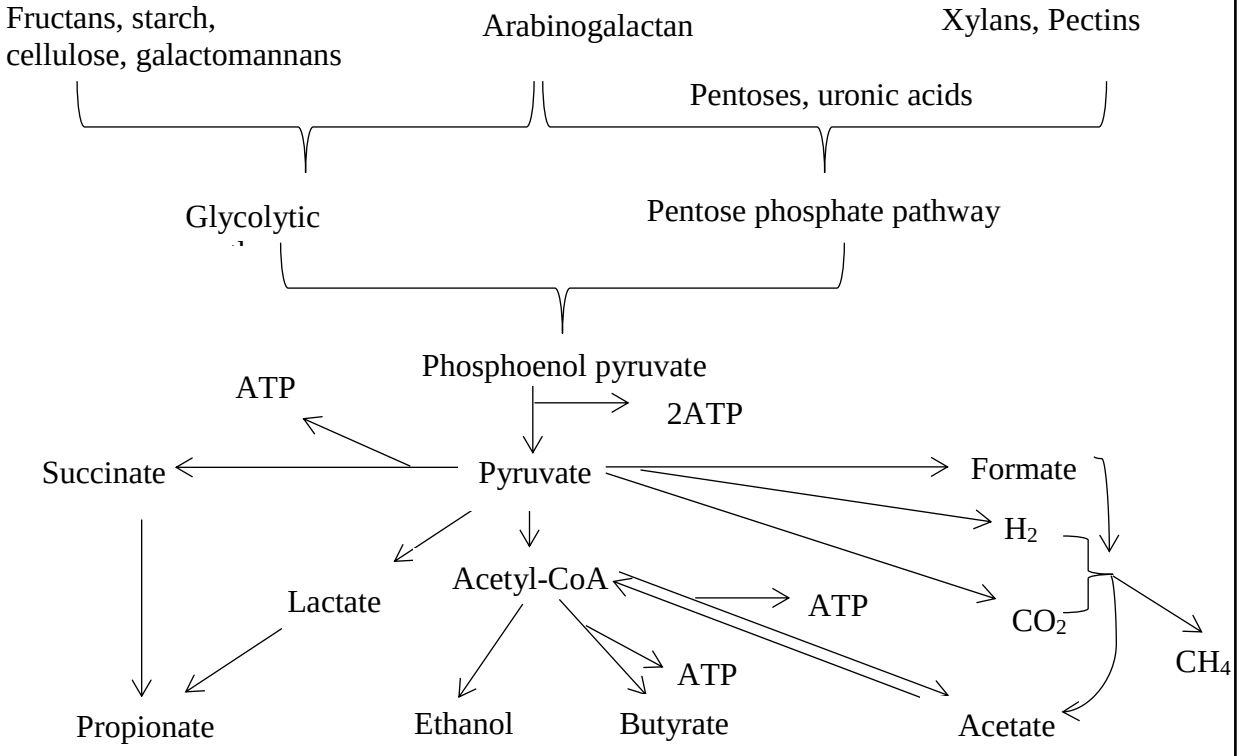

Figure 1.6 Schematic representation of the pathways for polysaccharide fermentation in the swine intestine (Bindelle et al., 2008)

1.6 Effect of feeding fiber on gut microflora, morphology, and nutrient digestion in pigs

The presence of dietary fiber has a substantial effect on changes in gut microflora populations (Awati et al., 2005). For example, the number of ileal bifidobacteria and enterobacteria in growing pigs were increased by the addition of guar gum or cellulose to the diet (Owusu-Asiedu et al., 2006). Another example is that pigs fed barley-based, high fiber diets had greater microbial activity in the hindgut of pigs compared with those fed low fiber diets (Jensen and Jørgensen, 1994). Dietary fiber also decreases rate of gastric emptying (Rainbird 1986) and induces earlier satiety (Rijnen et al., 2003). This helps to reduce hunger in limit-fed gestating sows.

Pigs fed high fiber diets also undergo changes in gut weight and morphology. The weight of the GIT of pigs fed high fiber diets increases (Kass et al., 1980; Stanogias et al., 1985; Anugwa et al., 1989), resulting in a reduction in pork carcass yield (Stewart et al., 2013; Asmus et al., 2014). This response has been shown when feeding high fiber diets containing DDGS (Asmus et al., 2014), SBH (Stewart et al., 2013), and wheat middlings (Stewart et al., 2013; Asmus et al., 2014), but no research has investigated the effects of specific dietary fiber components on dressing percentage. Pigs fed with high fiber diets also have increased villus height (Serena et al., 2008) and increased villus height to crypt depth ratio (Jin et al., 1994), but these general measures do not necessarily infer changes in digestive or absorptive capacity because dietary fiber also stimulates intestinal epithelial cell proliferation rate and increased cell turnover rate of pigs (Jin et al., 1994; Howard et al., 1995).

Feeding high fiber diets to pigs reduces energy and nutrient digestibility (Dégen et

al., 2007; Bindelle et al., 2008). The reduction in dietary GE digestibility is associated with a reduction in DM digestibility (Wilfart et al., 2007), with a 1% reduction in DE and ME content for each 1% increase in NDF concentration (Le Gall et al., 2009). However, the mechanism of how dietary fiber reduces energy and nutrient utilization is not very clear. Various researchers have suggested several potential mechanisms that may contribute to this response and include:

1) the cell wall structure of dietary fiber "traps" nutrients and prevents contact with digestive enzymes (Wenk, 2001)

2) soluble dietary fiber (e.g. guar gum and pectin) can slow digesta passage rate by increasing chyme viscosity to increase the ability of DM to retain water (Johansen et al., 1996; Le Goff et al., 2002; Owusu-Asiedu, 2006) and decrease enzyme contact, leading to reduced nutrient digestibility (Johnston et al., 2003; Chen et al., 2013)

3) increased digesta passage rate (Schulze et al., 1995) by feeding high fiber diets may reduce digesta retention time in the GIT

4) increased mucin secretion (Ito et al., 2009; Piel et al., 2005) induces greater endogenous losses of amino acids.

Researchers have observed that increased mucin secretion is accompanied by an increase in goblet cells because goblet cells secret mucins. An increase in goblet cell number coincides with an increase in intestinal surface area, but it is contradictory to the decreased nutrient digestion and absorption that occurs, because it has been often assumed that greater intestinal surface area corresponds to greater nutrient digestion and absorption area. Therefore, the cell differentiation, proliferation, and composition in the small intestine, especially changes in the number of enterocytes caused by feeding high

fiber diets, may explain part of the mechanism of reduced nutrient digestion and absorption.

Compared with our knowledge of the effects of dietary fiber on energy and nutrient digestion and absortion, very little information has been published on the effects of dietary fiber on mineral digestion and absorption in pigs. Some studies have shown that the addition of 6% cellulose depressed the apparent digestibility and absorption of Ca, P, Mg, and K (Girard et al., 1995). This may be because dietary fiber can shift the absorption of minerals, such as Ca and P, from the small intestine to the large intestine (Demigné et al., 1989). Some types of fiber may also bind some minerals in digesta and feces to reduce the absorption of minerals (Kritchevsky, 1988). In contrast, some studies have shown that the relatively low pH of rat large intestine, due to the production of VFA in animals fed high fiber diets, increases the solubility of some minerals such as Ca and P resulting in increased absorption (Rémséy et al., 1993). However, another study showed that the AID and ATTD of Ca, P, Mg, and Zn were not affected by the addition of 6% inulin to diets fed to pigs (Vanhoof and De Schrijver, 1996).

1.7 Dietary effects on cell proliferation and differentiation

Little is known about how diet modulates intestinal cell proliferation and differentiation. Most of the studies that have been published have focused on effects of high fat diets due to their relevance to human health. High fat diets induce obesity, which increases the number and function of LGR5+ intestinal stem cells of the mammalian intestine (Beyaz et al., 2016). In addition, consumption of high fat diets for 12 wk also affects enteroendocrine cell numbers by regulating hairy and enhancer of split-1 (*HES1*), Neurogenin 3 (*NGN3*), and Neurogenic differentiation 1 (*NEUROD1*; Sakar et al., 2014).

Feeding high protein diets also modifies intestinal cell differentiation where rats had increased intestinal cell proliferation rate by 50% (Sepulveda et al., 1982), increased villus height, crypt depth (Syme, 1982; King et al., 1983), and goblet cell activity (Lan et al., 2015) compared with those fed less protein.

1.8 Structure and physiological functions of the GIT and small intestine

The GIT is the location for digestion of feed and absorption of nutrients in swine, and includes the mouth, esophagus, stomach, pancreas, small intestine (duodenum, jejunum, and ileum), liver, gall bladder, and large intestine (cecum, colon, and rectum). The mouth is involved in mastication of feed to reduce particle size of feed and initiates break down of starch by the secretion of salivary amylase (Pedersen et al., 2002). The esophagus propels food from the mouth to the stomach, where food passes through the esophageal, cardiac, gastric, and pyloric areas (Campbell, 2009). Feed is mixed with hydrochloric acid, pepsinogen, and intrinsic factor in the stomach (Johnson, 1985), and then delivered to the small intestine, which consists of a tubular structure with openings for the entry of enzyme, bile, and other secretions from the liver and the pancreas (Tso and Crissinger, 2000). The small intestine serves as the main location of nutrient digestion by digestive enzymes and nutrient absorption (Dall and Moriarty, 1983). Undigested and unabsorbed food enters the cecum for fermentation and further breakdown by microflora. In the colon, absorption of VFA, electrolytes, and water, and bile salts occurs. Accumulated, undigested material present at the end of the colon is excreted through the rectum and anus (Dall and Moriarty, 1983). The structure of the small intestinal epithelium is showed in figure 1.7 (Barker, 2014), where it is arranged in

crypts and villi. Villi are composed of differentiated absorptive and secretory cells. The absorptive enterocytes (> 80%) are dominant in the villi. Secretory cells located in the villi include goblet cells (5-10%), endocrine cells (~1%), tuft cells (~0.4%), and membranous or microfold (M) cells (Gerbe et al., 2012). Crypts are composed of stems cells, transit amplifying (TA) cells, and Paneth cells (Barker, 2014). Paneth cells are the only secretory cells that are located in the crypt instead of villi.

Absorptive cells

Enterocytes

Intestinal enterocytes are columnar cells (Cheng and Leblond, 1974) and have digestive, absorptive, and barrier functions (Egberts et al., 1984; Smith, 1985). The surface of enterocyte membrane is folded into microvilli, forming a brush-border to enlarge the digestive and absorptive surface (Egberts et al., 1984). The brush-border membrane of the enterocytes secretes digestive enzymes such as aminopeptidase, sucrase (Gutschmidt et al., 1979), and lactase (Lojda et al., 1973). Enterocytes also absorb and

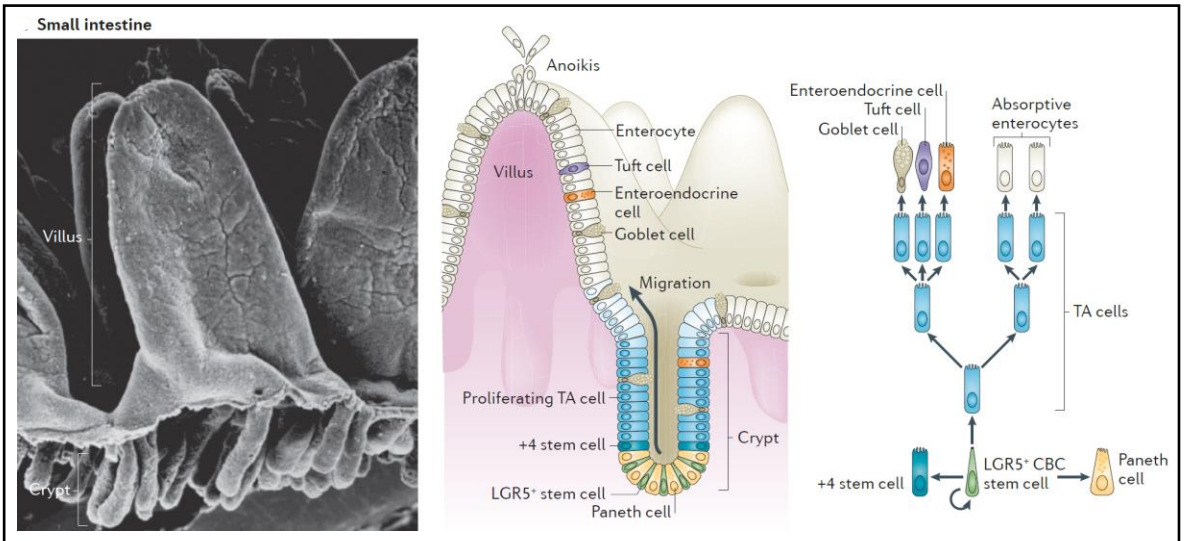

Figure 1.7 Epithelial self-renewal in the small intestinal epithelium (Barker, 2014)

degrade antigens, mainly through the action of enzymes and lysosomes, and translocate

the antigen across the epithelium or present the antigens to T cells within or beneath the epithelium (Snoeck et al., 2005).

Secretory cells

Goblet cells

Goblet cells are scattered throughout the epithelium and produce and maintain the protective mucus layer by synthesizing and secreting mucins, which are composed mainly of glycoproteins (Theodoropoulos, 2007). When weaned pigs (Piel et al., 2005) and rats (Ito et al., 2009) are fed diets containing viscous or soluble dietary fiber, the intestine secretes more mucin and the number of goblet cells were also increased.

Enteroendocrine cells

Enteroendocrine cells secrete gut hormones in response to nutrients in the intestine, and these hormones play a major role in the control of food intake and regulation of energy balance (Murphy and Bloom, 2006; Crosnier et al., 2006). Enteroendocrine cells secrete hormones including cholecystokinin (CCK), secretin, glucagon-like peptide-1 (GLP-1), GLP-2, peptide YY (PYY), ghrelin, glucose-dependent insulinotropic polypeptide (GIP), and gastrin among others (Furness et al., 2013).

M cells

M cells, also described as “lymphoepithelial cells” or “follicle-associated epithelial cells” (Wolf et al., 1984), are located over the surface of the gut-associated and bronchial-associated lymphoid follicles (Neutra, 1998). M cells are responsible for uptake of antigens and microorganisms through transepithelial transport from the lumen to the lymphoid system within the mucosa (Neutra et al., 1996). These cells interact closely

with immune cells of Peyer's patches and therefore, have a key function in the initiation of immunological response and tolerance (Kucharzik et al., 2000).

Tuft cells

Tuft cells are also referred to as "peculiar", "fibrilloversicular", "caveolated", or "brush" cells (Gerbe, 2011). These cells produce and secret opioids (Kokrashvili et al., 2009), and express taste-chemosensory components (Bezencon et al., 2008). Recently, tuft cells were shown to be the primary source of the parasite-induced cytokine, interleukin-25, and they promote type-2 immunity in response to intestinal parasites (Howitt et al., 2016; von Moltke et al., 2015).

Paneth cells

Paneth cells are granulated cells located at the bottom of small intestinal crypts. During maturation and differentiation, Paneth cells migrate downward to the bottom of the crypt and fill with numerous prominent apical cytoplasmic granules (Mathan et al., 1987). Paneth cells serve as multifunctional guardians of stem cells by secreting intestinal trefoil factor (Taupin et al., 1996), antimicrobial peptides (Poulsen et al., 1986), and also provide essential niche signals including epidermal growth factor (EGF), TGF-α, Wnt 3 and Notch-ligand delta 4 (Dll4; Sato et al., 2011).

1.9 Intestinal development, physiology, and biomarkers

In developmental biology, cellular differentiation refers to the process of a cell changing from one cell type to another (Slack, 2007). During cell growth, a less specialized cell type becomes a more specialized type with specific functions. The intestinal epithelium is a model of self-renewal (Barker, 2014). Intestinal cells are constantly generated from the multipotent stem cells (Lievin-Le Moal and Servin, 2006).

The overall renewal process involves intestinal stem cells giving rise to rapidly proliferating TA cells, which differentiate into the mature cells of the intestinal epithelium. The differentiated cells (both absorptive enterocytes and secretory cells) further migrate up to the villi and slough into the lumen (Barker, 2014; Gerbe et al., 2011). The cell precursors determine the differentiation pathway and cell type they will become because each of them can only differentiated into one type of mature cells (Figure 1.8). For example, a cell can either differentiate into a secretory or an absorptive cell lineage (Ogaki et al., 2013). Within the secretory lineage, a cell can either become a goblet, Paneth, or enteroendocrine cell (Shroyer et al., 2005). The decision of differentiation is regulated by cellular signal pathways (e.g. Notch and Wingless-Type (WNT) signaling pathways).

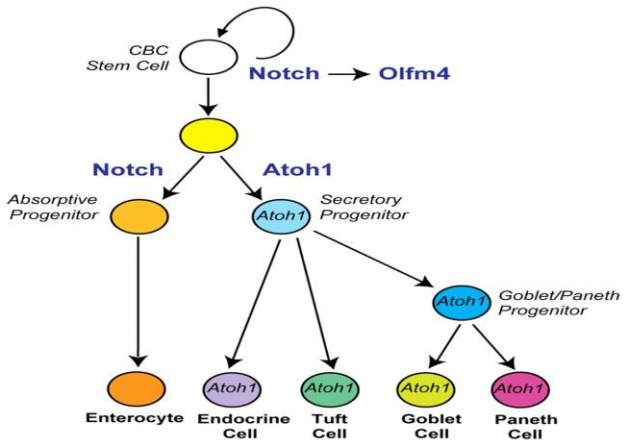

Figure 1.8. Notch signaling regulates several aspects of intestinal epithelial cell homeostasis (VanDussen et al., 2012)

Notch signaling pathway

The Notch signaling pathway regulates cell communication during animal development (Noah and Shroyer, 2013) through interaction of ligands and receptors. Notch ligands bind their receptors to the extracellular domain and induce the proteolytic cleavages of their receptors, releasing the intracellular domain (NICD). The released

NICD translocate to the cell nucleus and modify gene expression (Pellegrinet et al., 2011). There are 4 Notch receptors in mammals - Notch 1, Notch 2, Notch 3, and Notch 4, which are type I transmembrane proteins. These receptors have Delta like protein (DLL 1, DLL3, and DLL4) and Serrate like protein (JAGGED1 and JAGGED2) as their ligands in mammals. Notch signaling controls the fate of intestinal stem cells through its expressed receptors (Notch 1 and Notch 2) and ligands (DLL1, DLL4, and Jagged 1) in the crypts (Noah and Shroyer, 2013). Notch signaling can act on CBC stem cells directly to activate *OLFM4* transcription, maintain proliferation (VanDussen et al., 2012; Figure 1.8), and promotes cell differentiation to the absorptive cell lineage through repressing *ATOH1* transcription (Gerbe et al., 2011; Pellegrinet et al., 2011).

WNT signaling pathway

The WNT signaling pathway regulates cellular processes by stimulating intra-cellular signal transduction through cell surface receptors. The WNT pathway is activated by binding of a WNT protein ligand to a Frizzled family receptor (Komiya and Habas, 2008). Currently, 19 WNT proteins have been identified in humans and mice, and 10 Frizzled proteins have been identified in humans (Nusse and Varmus, 1992). There are β-catenin dependent and β-catenin independent WNT pathways. The WNT signaling controls cell fate along the crypt-villus axis (Clevers, 2006). Leucine-rich repeat-containing G-protein coupled receptor 5(LGR5) receptors interact with R-spondins to amplify WNT β-catenin signaling, maintain proliferation, and preserve stem cell properties (van Camp et al., 2014).

Proliferation of differentiated cells

Most intestinal epithelial cells have short life spans and must be replaced by continual cell proliferation in adult animals (Cooper, 2000). The entire epithelium generally renews within 4 to 7 days, except Paneth and endocrine cells (up to 57 days; Ireland et al., 2005). Therefore, continuous renewal of proliferating crypt progenitors is required to maintain the integrity of the epithelium.

Biomarkers in the cell proliferation and differentiation process

Biomarkers of cell proliferation

The Ki67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but not in resting cells (G0; Gerdes et al., 1984). Therefore, Ki67 is used as an indicator for the growth fraction of a given cell population (Alison, 1995). The proliferating cell nuclear antigen (PCNA) can replace Ki67 because it can be detected by immunohistochemistry in routinely-processed tissue. However, PCNA can be destroyed by prolonged fixation (48 h), and can be equally well visualized in all cells by aggressive antigen retrieval procedures.

Biomarkers of the crypt base columnar (CBC) stem cells

Leucine-rich repeat-containing G protein-coupled receptor 5-expression (*LGR5)* is the first identified marker for CBC stem cells (Barker et al., 2007). The *LGR5* gene behaves as a WNT target gene (Komiya and Habas, 2008). It is expressed in CBC cells in the small intestine (Barker et al., 2007), and therefore is used as a marker for CBC stem cells.

OLFM4 is another marker for CBC stem cells and is a direct target of Notch. *In situ* hybridization for *OLFM4* reveals a CBC-restricted expression pattern in the small intestinal epithelium (van der Flier et al., 2009).

biomarkers of the transit amplifying cells

SOX9 encodes a member of the sex-determining region Y family of transcription factors, which regulate cell proliferation in the intestine (Blache et al., 2004; Bastide et al., 2007). *SOX9* is expressed in stem cells, progenitor cells, and Paneth cells. The expression of *SOX9* requires a transcriptional effector of the WNT pathway (Blache et al., 2004). Inactivation of *SOX9* leads to disappearance of Paneth cells and a decrease of a goblet cell lineage (Bastide et al., 2007).

Delta like 1 (*DLL1*) and *Delta like 4* (*DLL4*) are expressed in the same intestinal secretory cells. They are ligands for Notch signaling pathway, which is essential for controlling the fate of intestinal stem cells (Sander and Powell, 2004). Mutation of both *DLL1*and *DLL4* reduced proliferation and forced CBC stem cells to differentiate into secretory cells, as predominantly goblet cells (Pellegrinet et al., 2011). Therefore, Delta genes are mediators that restrict cells from committing to a secretory fate.

HES1 is a basic helix-loop-helix transcription factor and an absorptive progenitor. Activation of Notch/HES1 signaling promotes differentiation of the absorptive lineage and decreases differentiation of the secretory lineage (Ogaki et al., 2013).

Atonal bHLH transcription factor 1 (*ATOH1*), also termed MATH1 or HATH1, is a basic helix-loop-helix transcriptional activator that is also expressed in secretory progenitor or secretory cells and it is essential for secretory cell differentiation (Mulvaney and Dabdoub, 2012). ATOH1 is repressed by HES1 (Zheng et al., 2011), ATOH1, and Notch/HES, which play opposing roles in promoting secretory versus absorptive epithelial cell types.

Biomarkers of differentiated cells

Enterocyte biomarkers

The fatty acid binding protein (FABP) superfamily is comprised of 14 to 15 kDa soluble proteins which bind with a high affinity to either long-chain fatty acids, bile acids, or retinoids (Besnard et al., 2002). In the small intestine, two different FABP are expressed: the intestinal (I-FABP), and the ileal bile acid binding protein (I-BABP). I-FABP is also called FABP2, which is abundantly present in fully differentiated small intestinal enterocytes (Levy et al., 2009), and is widely used as a marker of enterocytes (Gajda and Storch, 2015; Reisinger et al., 2014).

Goblet cell biomarkers

Mucins (MUC) are O-glycosylated proteins that protect the epithelial surface (Theodoropoulos and Carraway, 2007). Categories of mucins have been suggested by Theodoropoulos and Carraway (2007) based on their functions and include membrane mucins that have a transmembrane sequence, gel-forming mucins that are secreted and found in mucus gels, and others (e.g. MUC7, MUC8, MUC9, MUC10, MUC11, MUC14, MUC19). Membrane mucins, MUC1 and MUC4, are widely expressed in many tissues, but MUC2 is secreted by goblet cells and is mainly present in the intestine. As a result, MUC2 is widely used as a marker for goblet cells (Grün et al., 2015; McIntire et al., 2011).

Enteroendocrine cell biomarkers

Chromogranin A (CHGA) is produced in the adrenal medulla and secreted with catecholamines (Cohn et al., 1982). It is an acidic glycoprotein that belongs to granin family and is secreted by endocrine and neuroendocrine cells (Cohn et al., 1984). The functions of CHGA include being a precursor of some bioactive peptides (e.g. parastatin)

that inhibit parathyroid secretion (Fascioto et al., 2000), promote granule biogenesis (Stettler et al., 2009), and regulate calcium homeostasis (Yoo et al., 2010). Because CHGA is commonly secreted in most of the endocrine cells, it is widely used as a marker to identify endocrine cells (Zhong et al., 2015; Grün et al., 2015).

Paneth cells marker

Lysozyme (LYZ) was initially reported as an enzyme capable of lysing bacteria (Fleming, 1922). It also has antiviral function (Oevermann et al., 2003; Lee-Huang et al., 2005) and works as a cellular and humoral defense stimulator (Siwicki et al., 1998). Lysozyme is encoded by *LYZ* gene and *LYZ1* and has been widely used as Paneth cell marker in small intestine (Zhong et al., 2015; Wang et al., 2011; Grün et al., 2015).

Table 1.5. Genes evaluated in this thesis and their functions

Item	Full name	Gene expression location	Gene function	Reference
Differentiated cell lineage				
FABP2	Intestinal fatty acid binding protein	Small intestine enterocytes	Marker of enterocytes	Gajda and Storch, 2015; Reisinger et al., 2014
MUC2	Mucin 2	Goblet cells	Marker of Goblet cells	McIntire et al., 2011
LYZ1	Lysozyme 1	Paneth cells	Marker of Paneth cells	Zhong et al., 2015
CHGA	Chromogranin A	Endocrine cells	Marker of Endocrine cells	Zhong et al., 2015
Progenitors				
LGR5	Leucine rich repeat containing G protein-coupled receptor 5	CBC stem cell	WNT target gene; Progenitor cell activator	Komiya and Habas, 2008; Barker, 2007
OFLM4	Olfactomedin 4	CBC stem cell	Progenitor cell activator	van der Flier et al., 2009
HES1	Hairy and enhancer of split-1	Progenitor cells	Absorptive progenitor	Ogaki et al., 2013
DLL4	Delta-like 4	Secretory cells	Secretory inhibitor	Sander and Powell, 2004
ATOH1	Atonal homolog 1	Secretory progenitor or secretory cells	Secretory progenitor	Mulvaney and Dabdoub, 2012

1.10 Energy evaluation and diet formulation systems for high fiber ingredients

Energy is the most expensive component in swine diets and represents as much as 67% of total diet cost. Therefore, accurate estimation of dietary energy is very important to optimize caloric efficiency of swine diets. However, increasing the fiber inclusion rate in swine diets leads to an increase in the difference between gross energy (GE) and net energy value of diets (Dégen et al., 2007) due to the interactive effects of dietary fiber with other nutrients. For example, DE fails to consider losses of energy associated with digestion and metabolism of feed and therefore, overestimates the energy value of high-fiber feedstuffs compared with low-fiber feedstuffs (NRC, 1996). As a result, more accurate assessments of the DE, ME, and NE contributions of various types and amounts of fiber in swine diets need to be investigated.

Energy systems

There are 4 energy measurement systems used in animal nutrition: GE, DE, ME, and NE. Historically in the U.S., the DE and ME systems were predominantly used to evaluate feed ingredients and formulate swine diets. However, *in vivo* determination of DE and ME content of feed ingredients is time consuming, expensive, and estimates only accurately apply to the sources of test ingredients evaluated. Book values from published references are available and simple and convenient to use, but lack of accuracy when sources of ingredients have high variability in energy and nutrient composition. Prediction equations (Noblet and Perez, 1993) and commercial nutrient determination systems (e.g. Cargill and InctraPorc®) may be more accurate, but large databases are needed. These systems have been relatively accurate for characterization of the actual energy value of low fiber, corn and soybean meal based diets, but overestimate energy

value when high fiber ingredients are fed. As a result, increased use of high fiber ingredients in U.S. swine diets has led nutritionists to adopt modified-ME or NE systems to achieve a more accurate assessment of the true utilizable energy value. Unfortunately, due to the complexity, need for specialized equipment, time, and cost, *in vivo* determination of NE content of various feed ingredients has been limited. Therefore, there is a tremendous need to develop alternative methods for quantifying the utilizable energy portion of feed ingredients and develop a more robust data base of DE, ME, and NE values that can be used in practical swine diet formulation.

Methods to estimate DE, ME, and NE

Near infrared reflectance spectroscopy (NIRS)

Spectroscopy literally means looking at light and is based on the interaction of electromagnetic radiation with the matter to be analyzed (Givens et al., 1997). Near infrared light is defined as the wavelength region from 730-2500 nm (Osborne and Fearn, 1986). Near infrared reflectance spectroscopy (NIRS) is a non-consumptive, instrumental method for fast, accurate, and precise evaluation for nutritional quality of a wide range of feed ingredients, including cereal grains and oilseeds, and does not require chemical reagents, or produce fumes or waste products (Givens et al., 1997). Once the calibration is developed, this technique is relatively simple and does not require extensive training for the operators. The usual procedure for calibrating involves selection of representative samples, acquiring spectra and reference analyses, and statistical modeling and validation (Figure 1.9).

The NIRS technique relies on chemometrics. Each of the major organic feed components has absorption characteristics (due to vibrations arising from the stretching

and bending of H bonds associated with C, O, and N) in the near infrared region that are specific to the chemical component. These absorption characteristics primarily determine diffuse reflectance, which provides the means for assessing composition. The NIRS technique has been used to predict chemical composition of many ingredients (Park et al., 1998). It has also been used to predict DE and ME of corn DDGS (Zhou et al., 2012). Unfortunately, the calibrations developed by Zhou et al. (2012) referenced DE and ME values that were calculated from ingredient chemical composition using equations derived for diets by Noblet and Perez (1993). These equations were specifically developed for diets and not for feed ingredients, and likely do not accurately represent actual *in vivo* values. The spectral model of NIRS also showed a greater prediction power for ME than using chemical components or an *in vitro* digestion method of wheat and barley fed to sheep (Deaville et al., 2009). However, the NIRS method requires a large data set representing a large number of highly variable samples to develop accurate calibrations. Furthermore, calibrations must be updated periodically, particularly when substantial changes in the chemical composition of various sources of an ingredient change. There is potential for using NIRS to estimate DE, ME, and NE content of feed ingredients, but validation of the data and the calculation process is very complicated and has not been developed for practical application yet.

Prediction equations

Energy prediction equations using analyzed chemical composition data for feed and feed ingredients can be an accurate, inexpensive, and a fast method of obtaining dynamic estimates for use in feed formulation. Prediction equations have been developed by Noblet and Perez (1993) to estimate DE, ME, and NE content using chemical

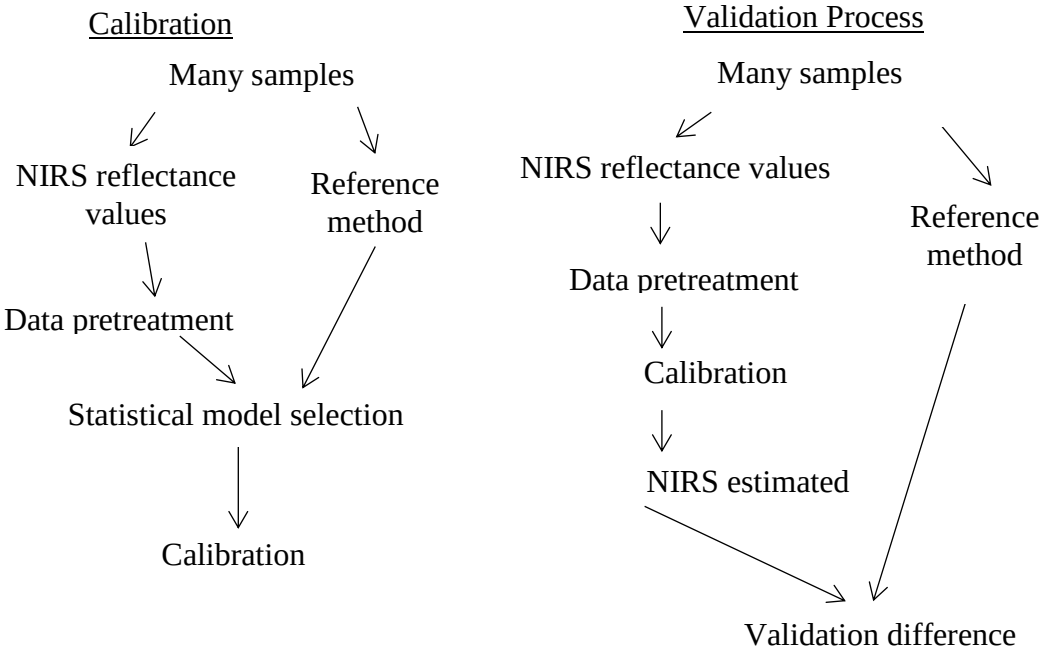

Figure 1.9 Calibration and validation process for NIRS (Leeson et al., 2000)

composition of 114 diets, but not for specific ingredients. Noblet and Jaguelin-Peyraud (2007) also developed DE and NE of feed ingredients from prediction equations based on *in vitro* organic matter (OM) digestibility and chemical composition. Recognizing the limitations of use of inaccurate estimates for some ingredients (Urriola et al., 2014), various researchers have developed DE and ME prediction equations for various feed ingredients, such as corn DDGS (Pedersen et al., 2007; Anderson et al., 2012; Urriola et al., 2014). Although some equations predict DE and ME content with reasonable accuracy and precision, they still have inherent prediction error and bias because they are based on chemical composition, and likely do not account for the complex interactions among chemical components that occur during the digestive process of nutrients *in vivo*. Other energy prediction equations have been developed using digestible nutrients to estimate NE content (French Institut National de la Recherche Agronomique, INRA; Dutch Central Bureau Livestock Feeding, CVB; and Danish potential physiological

energy (PPE) system) to increase the accuracy of prediction. However, these systems require measurement of digestible nutrients, which requires conducting expensive and time consuming *in vivo* animal experiments. Therefore, there is a need to develop and validate the accuracy of using alternative *in vitro* methods to predict DE, ME, and NE content and nutrient digestibility of ingredients.

The three-step in vitro method

In vitro nutrient digestibility methods originated from ruminant studies (Tilley and Terry, 1963), and have been modified and developed for evaluating feeds and feed ingredients for monogastric animals. Initial methods focused on *in vitro* protein digestion by incubating feeds or feed ingredients with pepsin (Sheffner et al., 1956), trypsin (Saunders et al., 1973), papain (Buchanan, 1969), pronase (Taverner and Farrell, 1981) or rennin (Bhatty, 1982). Later, improved methods were developed to simulate more complex *in vivo* digestion processs in the stomach and small intestine by using pepsin with jejunum-fistulated pigs (Furuya et al., 1979), or pepsin with pancreatin (Büchmann, 1979). After that, hindgut fermentation was considered in the *in vitro* system by applying rumen liquor (Vervaeke et al., 1989) or viscozyme (Boisen and Fernández, 1997) to determine total tract dry matter or OM digestibilities of feed ingredients.

The three-step procedure developed by Boisen and Fernández (1997) has become the most widely used *in vitro* procedure to evaluate DM or OM digestibility of feed ingredients for swine. Briefly, about 0.5 g of ground samples are subjected to pepsin incubation for 2 h, pancreatin incubation for 4 h at 39℃, and viscozyme (including arabinase, cellulase, β-glucanase, hemicellulase, xylanase and pectinase) inoculation for 18 h. The residues remaining are filtered, dried, and weighed to calculate *in vitro* total

tract DM digestibility. This method has been used predict *in vivo* DM digestibility and energy digestibility of barley (Regmi et al., 2008) and wheat (Regmi et al., 2009). Later, Bindelle et al. (2007) used fecal bacteria collected from swine to replace viscozyme for large intestine inoculation, and collect gas produced during the process. This modified three-step procedure has been used widely to investigate the fermentation characteristics of diets and ingredients by developing gas production curves (Bindelle et al., 2009, Jha et al, 2011a, Jha et al., 2011b). Therefore, the three-step procedure and modified three-step procedure appear to be promising tools to predict *in vivo* nutrient digestibility as predictors of DE and ME for ingredients used in swine diets.

Computer-controlled simulated digestion system (CCSDS)

Traditional *in vitro* digestion techniques are conducted manually and require cumbersome management of pH, addition of digestive enzymes and separation of digested and undigested substances, which introduce error and may contribute to low repeatability and imprecise results (Zijlstra, 2006; Losada et al., 2010). As a result, a novel *in vitro* CCSDS with simulated small intestinal fluid containing amylase, trypsin, and chymotrypsin was developed to evaluate energy and nutrient digestibility of feed ingredients for poultry and swine (Zhao et al., 2014a; Zhao et al., 2014b; Chen et al., 2014). The CCSDS is composed of 5 digestion chambers (Figure 1.10), 2 single-channel peristaltic pumps, 10 electronic valves, a multiple-channel peristaltic pump, a warmed-air shaking incubator, a cooled-air incubator, a water bath incubator, a decompression tube, 4 reagent bottles, 3 buckets, a single-chip microcomputer, a computer, and control software

Overall, none of the *in vitro* methods are perfect to estimate DE, ME, or NE. However, these methods provide an alternative way to utilize the feed ingredients more

efficiently, but improvements are still needed to increase the accuracy of those methods.

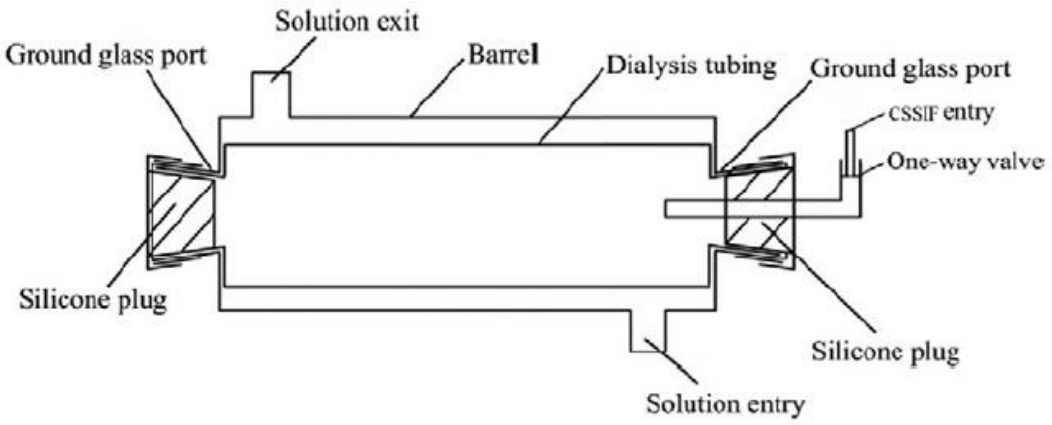

Figure 1.10 Schematic diagram of digestion chamber. CSSIF = concentrated simulated small intestinal fluid (Zhao et al., 2014a)

1.11 Production and utilization of DDGS, soybean hulls, and wheat straw in swine diets

Production of DDGS

In 2015, fuel ethanol production in the U.S. was estimated at 14,700 million gallons and an estimated 37 million metric tons of DDGS was produced, of which 15% was consumed by swine (RFA, 2016). There are two main sources of DDGS including traditional sources from beverage distilleries, but the majority is produced by fuel-ethanol plants. Corn DDGS is the primary co-product of fuel ethanol production from the dry-grind process (Rosentrater, 2012). A detailed flow chart of the dry-grind process is shown in Figure 1.11, and is described by Rosentrater et al. (2012). Briefly, whole corn is ground with a hammer mill, and water is added to the ground corn to create a slurry, which may be cooked. Liquefaction occurs and carbohydrase enzymes and yeast are added to saccharify the mash and initiate the fermentation which converts starch to ethanol. After about 40-72 hours of fermentation, the ethanol is distilled from the

fermented mash, and the residual whole stillage is separated into thin stillage and wet grains. Corn oil may be extracted from the thin stillage and water is removed by centrifugation to produce condensed distillers solubles. Finally, 75 to 100% of the condensed distillers solubles is blended with the wet grains fraction and dried using rotary dryers to produce DDGS.

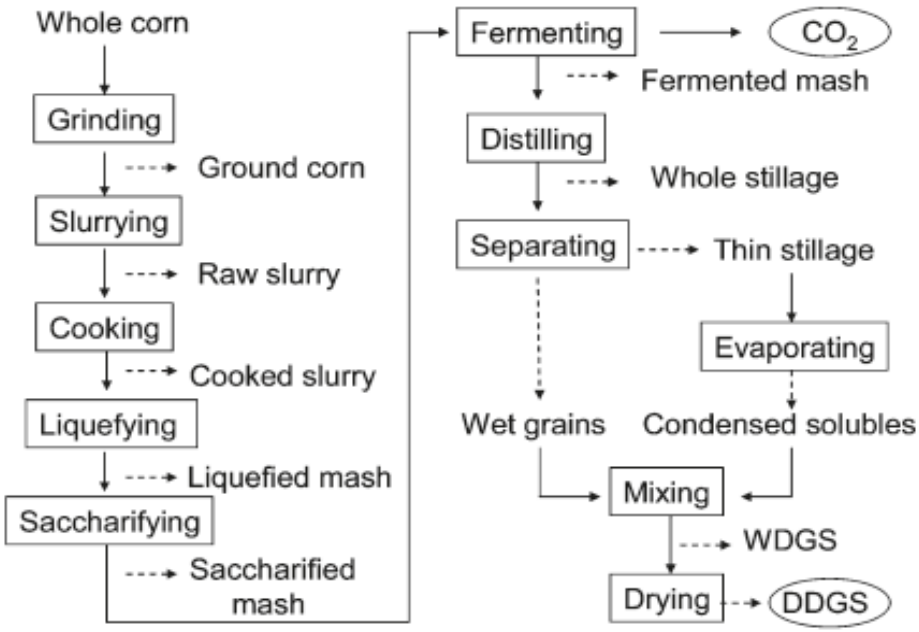

Figure 1.11 Schematic diagram of a conventional dry-grind ethanol production from corn (Liu, 2011). DDGS: distillers dried grains with solubles, WDGS: wet distillers grains with solubles

Use of DDGS in swine diets

The use of DDGS in swine diets has increased dramatically over the past 15 years, where it serves as an energy, digestible amino acid, and phosphorus source to partially replace some of the corn, soybean meal, and inorganic phosphorus supplements. The inclusion rate of corn DDGS may be up to 20-30% in weanling pig diets, and up to 45% in growing-finishing pig diets without affecting ADG of the pigs (Graham et al.,

2014b). For lactating sows, up to 40% DDGS diets can be fed without reducing sow or litter performance (Wang et al., 2013; Baidoo et al., 2014), while up to 40-50% DDGS diets can be fed successfully to gestating sows (Wang et al., 2013). However, one of the major limitations affecting DDGS use and inclusion rates in swine diets is the highly variable ME, NE, and nutrient content among sources (Stein and Shurson, 2009), which is caused by several factors in the ethanol and co-product production process (Olentine, 1986). Furthermore, corn oil extraction technologies have been implemented in the majority of U.S. ethanol plants, which has created more variable ether extract content, ranging from 5 to 14% crude fat. The NRC (2012) provides energy and nutrient composition data based on categories of ether extract concentration (> 10%, 6-9%, and < 4%). The other nutrient components of DDGS are also vary variable (Table 1.6). Unfortunately, at the time of publication of this reference, there were very little published data on nutrient composition and DE, ME, and NE content of the medium and low oil DDGS sources, creating uncertainty regarding the accuracy of these estimates. Furthermore, studies by Anderson et al. (2012) and Kerr et al. (2013) showed that ether extract concentration of the DDGS is not a good predictor DE and ME content in DDGS. This lack of accurate prediction from ether extract content appears to be partially due to the high variability in ether extract digestibility among DDGS sources (Kerr et al., 2013). In addition, the relatively high and variable concentration of fiber and variable fiber digestibility (29.3 to 57.0%; Urriola et al., 2010) appears to be an important factor that affects the ME content of DDGS sources. Therefore, accurate estimation of fiber content and digestibility is important to obtain accurate estimates of ME content of various DDGS sources fed to pigs.

Table 1.6 Nutrient composition of corn distillers dried grains with solubles (DDGS, > 6 and < 9% oil), DM basis (Feedipedia, 2015; NRC, 2012)[1]

Item	Mean	SD	Min	Max	Number of samples
DM, %	89.0	1.4	86.6	91.9	332
CP, %	29.5	1.8	25.2	33.5	347
Crude fiber, %	7.9	0.9	6.0	9.9	228
NDF, %	34.2	6.8	18.3	47.4	113
ADF, %	13.6	4.2	7.9	25.1	143
Lignin, %	4.3	1.9	1.0	8.4	32
Ether extract., %	11.1	2.2	7.1	15.7	265
Ash, %	5.4	1.0	3.4	7.5	283
Starch, %	9.3	3.0	3.9	15.2	121
Total sugars, %	1.7	1.4	0.2	4.8	16
GE, kcal/kg	4710				
DE, kcal/kg	3582				
ME, kcal/kg	3396				
NE, kcal/kg	2343				

[1]Nutrient concentrations are referenced from Feedipedia (2015), energy values are referenced from NRC (2012)

Production of soybean hulls (SBH)

Soybean processing (Figure 1.12) involves a series of steps to produce commodities for food, industrial, and animal feed uses. Soybean hulls (SBH) are a by-product of soybean processing and are used for animal feed (Kornegay, 1978). Generally, soybean meal processing involves cleaning, cracking and removal of the hulls before solvent extraction of soybean oil. The hulls can be toasted, ground, and blended back into soybean meal to produce a 44% crude protein meal, or sold separately as SBH (Smith et al., 1977).

Utilization of SBH in swine diets

Soybean hulls are high in fiber content, and NDF ranges from 53 to 72% (Table 1.7. The amount of SBH used in swine diets is much less than DDGS because of their relatively high fiber content, along with low protein and ether extract content. Several

research studies have evaluated the effects of feeding diets containing SBH on energy and nutrient digestibility and growth performance in growing pig diets (Table 1.8). Studies have shown that feeding SBH to growing-finishing pigs increases their large intestine (Whitney et al., 2006), empty GIT weight (Rijnen et al., 2001), fecal nitrogen (Zervas and Zijlstra, 2002), DM excretion (Kornegay, 1978), and reduced ATTD of ADF (Kornegay, 1978). Feeding SBH also reduces body weight (Whitney et al., 2006), average daily feed intake (ADFI), and average daily gain (ADG; Kornegay et al., 1995), DE, ME, and ATTD of nutrients (Kornegay, 1978). Of all swine production phases, SBH are most widely used as a fiber source in diets for gestating sows to increase bulk which is useful for increasing satiety of gestating sows and reducing the stereotypic behaviors (Ramonet et al., 2000) .

Table 1.7 Nutrient composition of soybean hulls (SBH), DM basis (Feedipedia, 2011; NRC, 2012)[1]

Item	Mean	SD	Min	Max	Number of samples
DM, %	89.1	1.0	87.0	91.8	795
CP, %	13.2	1.8	10.5	19.2	761
Crude fiber, %	38.8	2.7	30.7	43.6	793
NDF, %	64.1	4.3	53.4	72.2	148
ADF, %	45.8	2.9	39.5	51.9	141
Lignin, %	2.4	0.7	1.2	4.4	171
Ether extract., %	2.2	0.9	1.0	5.2	453
Ash, %	5.3	0.3	4.6	5.9	428
Starch, %	5.5	3.5	0.3	9.8	13
Total sugars, %	1.6	1.3	0.3	4.0	7
GE, kcal/kg	4210				
DE, kcal/kg	2008				
ME, kcal/kg	1938				
NE, kcal/kg	989				

[1]Nutrients concentration are referenced from Feedipedia (2011), energy values are referenced from NRC (2012)

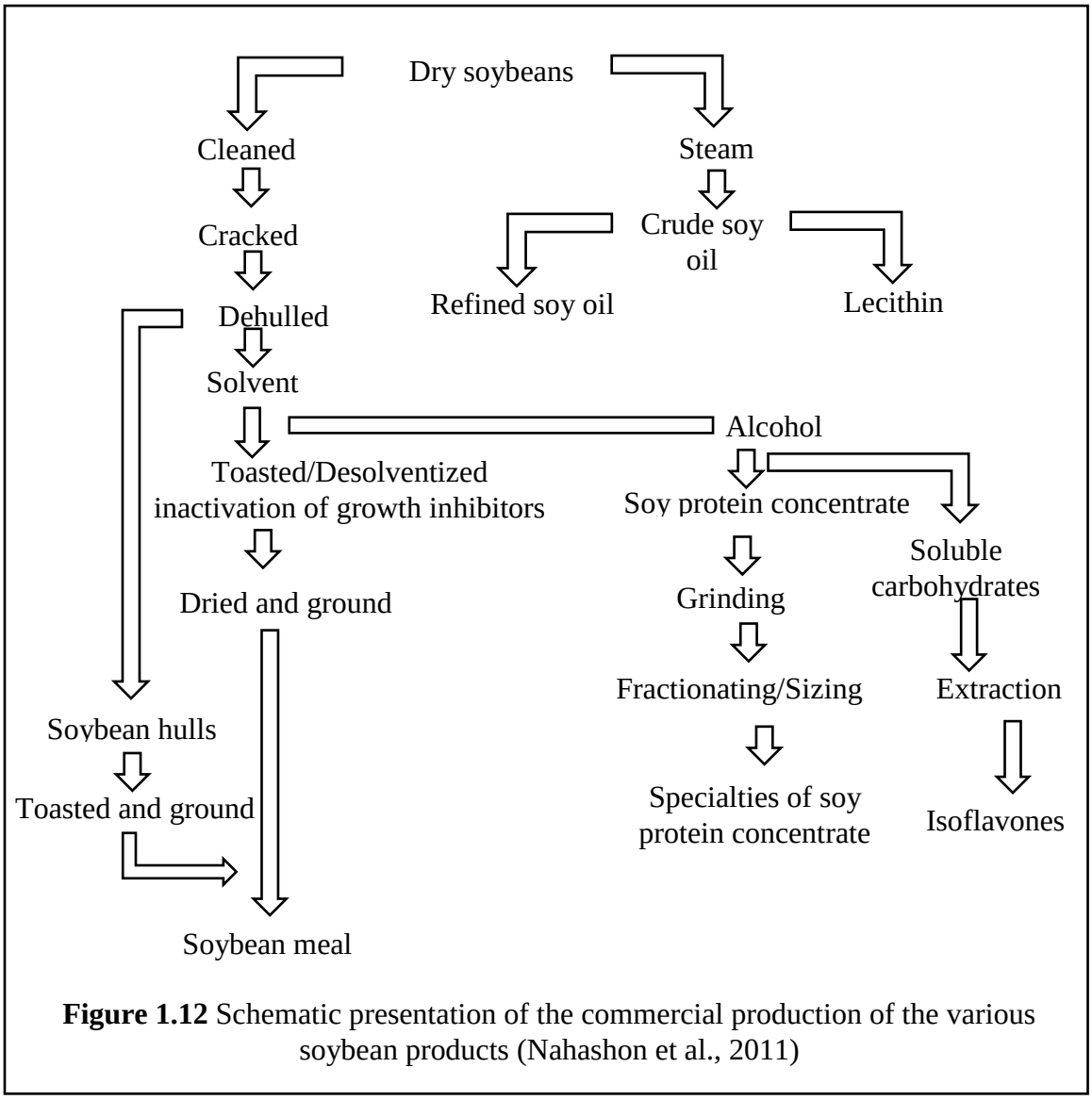

Figure 1.12 Schematic presentation of the commercial production of the various soybean products (Nahashon et al., 2011)

Table 1.8 Summary of published studies when including soybean hulls (SBH) in swine diets

Reference	SBH inclusion rate	BW, kg	Exp. length, d	Effect of growth performance and other responses
Kornegary (1978)	0, 15, 30%	46.3	30	Increased feces DM output; decreased urine volume Decreased DE, ME, ATTD of DM, CP, ash, EE, NFE[7]; increased ATTD of ADF, and nitrogen retention as percentage of digested.
Kornegary (1981)	0, 7.5, 15, 30%	Gestating sows: 150.5	-	Increased feces DM output; decreased wet feces DM Decreased ATTD[6] of DM, DE, and CP; Increased ATTD of ADF
Cho et al. (1985)	0, 8%	12	60	Decreased the cholesterol-induced hypercholesterolemia
Kornegay et al. (1995)	0, 8%	7.8 - 41.8	35	Numerically increased ADFI by 9.8% in starter phase but decreased ADFI by 25.9% and ADG by 13.5% in grower phase
Kendall et al. (1999)	0, 10%	60	63	Decreased ADG, Gain:Feed, total nitrogen and nitrogen in manure
Bowers et al.(2000)	0, 3, 6, 9%	68-125	49	Decreased ADG[1] and Gain:Feed; increased loin depth and lean percentage
DeCamp et al. (2001)	0, 10%	82-121	42	Increased ADG, Gain:Feed, adjusted final backfat, total nitrogen, ammonium nitrogen and total VFA[2] in manure
Rijnen et al. (2001)	4, 15, 26, 37, 48%	63.0	42	Increased weight of empty GIT, stomach, and colon
Dilger et al. (2004)	0, 3, 6, 9%	35	49	Linearly decrease AID of DM, GE and several AA
Chee et al. (2005)	0, 6, 10, 12%	16-35	42	No significant differences in growth performance, back-fat thickness and adjusted loin eye muscle area
Whitney et al. (2006)	0, 5%	5.6	42	Reduced BW by 13.3% and increased large intestine weight
Jacela et al. (2007)	66.7 %	68	-	Two types of SBH showed different energy values (DE, ME, and estimated NE) and SID[5] of Lys, Met, and Thr
Wang et al. (2009)	0, 5, 10, 15%	55	28	ATTD of DM, blood urea nitrogen, slurry pH and ammonia emissions were linearly decreased by the addition of SBH
Dégen et al. (2011)	0, 2.5, 5, 7.5, 10%	35	28	Adding 2.5% SBH depressed the AID[3] of the most AA
Stewart et al. (2013)	0, 30%	25-57; 85-126	-	Decreased ADG and Gain:Feed in growing phase; Depressed dressing percentage and chilled carcass in both growing and finishing phase
Falomo (2015)	0, 20%	68-125	49	Numerically decreased ADFI[4] by 13.1% and ADG by 14%
Schertz (2015)	0, 20%	61	49	Numerically increased ADFI by 5.2% but decreased ADG by 8.9%

[1]ADG = average daily gain

[2]VFA = volatile fatty acids
[3]AID = apparent ileal digestibility
[4]ADFI = average daily feed intake
[5]SID = standard ileal digestibility
[6]ATTD = apparent total tract digestibility
[7]NFE = nitrogen-free extrac

Production and utilization of wheat straw (WS) in swine diets

Wheat straw is a by-product of the plant residue remaining after harvesting wheat grain, and is primarily used as bedding material in livestock housing systems because of its high fiber and low ME and NE, crude protein, and ether extract content (Table 1.9). As a result, very little is used in practical growing-finishing swine diets because of its inferior nutritional value and negative impacts on energy and nutrient utilization and growth performance (Table 1.10). Feeding straw has a negative effect on energy digestibility in growing pigs, where inclusion of 10 to 15% wheat straw for finishing pigs decreased the digestibility of protein, NDF, hemicellulose and gross energy (Falkowska et al., 2006). However, like SBH, it has been used as as a forage substrate by serving as a diluent in feed to control energy intake and weight gains, increase satiety, and reduce negative stereotypic behaviors of gestating sows (Spoolder et al., 1995). Supplying gestating sows with a diet containing WS was found to be effective in preventing bar biting and chain manipulations and reduced stereotypic behavior (Stewart et al., 2011). In gestatign sows, dietary WS improved sow and litter performance by increasing litter size and total litter weight at birth and weaning (Veum et al., 2009).

Table 1.9 Nutrient composition of wheat straw (WS), DM basis (Feedipedia, 2014)

Item	Mean	SD	Min	Max	Number of samples
DM, %	91.0	1.3	87.3	93.8	438
CP, %	4.2	0.7	2.6	6.0	428
Crude fiber, %	41.5	2.1	36.6	46.2	438
NDF, %	77.5	4.2	65.4	86.0	85
ADF, %	50.0	3.5	43.3	57.0	80
Lignin, %	7.2	1.0	5.3	9.7	203
Ether extract, %	1.4	0.5	0.7	2.8	53
Ash, %	6.7	1.2	4.4	10.0	433
Starch, %	1.0	0.6	0.1	2.6	114
Total sugars, %	1.2	0.9	0.3	5.7	138
Gross energy, kcal/kg DM	4419	0.6	16.0	18.5	18

Table 1.10 Use of wheat straw (WS) in swine diets

Reference	WS inclusion	Pigs BW, kg	Exp. length, d	Effect of growth performance and other response
Woodman and Evans (1947)	0, 25%	36-106	85	Reduced the carcass percentage
Forbes and Hamilton (1952)	14.4%	56		Reduced the digestibility of energy
Chabeauti et al. (1991)	0, 22.13%	35	20	Reduced digestibility of nitrogen, energy, NDF and NSP compared with control diet, wheat bran or soybean hulls diets
Jin et al. (1994)	0, 10%	14	14	Decreased tissue (jejunum, ileum and liver) concentrations of DNA, increased content of RNA in colon, number of crypt cell nuclei and epithelial cells, as well as width of intestinal villi and depth of intestinal crypts
Mariscal-Landín et al. (1995)	0.5, 1, 3%	35	42	Increased the endogenous losses of nitrogen and AA
Spoolder et al. (1995)	1.5 kg/d	Gestating sows	77	Reduced the development of excessive chain and bar manipulation in food restricted sows
Yan et al. (1995)	17.4%	Gestating sows	74	Increased feces output and reduced digestibility of DM, NSP[5] and individual constituent sugars.
Whittaker et al. (1998)	1.5 kg/d	Gestating sows	77	Reducing development of stereotypic behavior
Hakansson et al. (2000)	0, 8, 16%	24-105	90	WS diets linearly decreased the digestibility of CP, OM, and GE; increased ADFI[1] and reduced ADG[2]
Rijnen et al. (2001)	15%	55.3	42	Increased empty weight of stomach and total GIT[4] by 6.7%
Renteria-Flores et al. (2008)	11.64%	Gestating sows	98	Reduced digestibility of energy, total nitrogen and IDF[3] compared with control, oat hull, or sugar beet pulp diets
Veum et al. (2009)	13.35%	Gestating sows	-	Improved litter size and total litter weight at birth and weaning of 0.51 pig/litter, and total litter weight increases of 0.87 kg at birth and 3.59 kg at weaning compared with control sows and litters
Stewart et al. (2011)	Free access	Gestating sows	-	Less chain-chewing and bar-biting behavior

[1]ADFI = average daily feed intake
[2]ADG = average daily gain
[3]IDF = insoluble dietary fiber
[4]GIT = gastrointestinal tract
[5]NSP = non-starch polysaccharides

Fiber composition and variability of corn DDGS, SBH, and WS

Different dietary fiber sources have different fiber composition and unique fiber characteristics. Corn DDGS, SBH, and WS can be considered as feed ingredients with a high proportion of insoluble dietary fiber (IDF) compared withTDF: corn DDGS (IDF: TDF = 95 to 100%; Urriola et al., 2010), SBH (IDF: TDF = 83 to 94%; Cole et al., 1999), and WS (IDF: TDF = 98 to 99%; Panthapulakkal et al., 2006; Alemdar and Sain, 2008). However, the fiber composition among the 3 high fiber ingredients is different (Table 1.11). The WS includes greater concentration of TDF, NDF, ADF, lignin, and crude fiber than corn DDGS and SBH. The SBH also includes greater concentration of TDF, NDF, ADF, and crude fiber than corn DDGS. Because of these fiber composition differences, we chose these 3 feed ingredients to investigate the physiologic effects of different dietary fiber ingredients and sources in our studies.

Even though dietary fiber is unique among feed ingredients due to the nutritional-based definition, it must be measured chemically (Mertens, 2003). As stated in section 1.3, soluble fiber fractions as well as insoluble fiber fractions can be measured with the TDF procedure (Method 991.43; AOAC, 2006). The NDF (sum of cellulose, hemicellulose, and lignin) and ADF (sum of cellulose and lignin) can be measured by the detergent fiber procedure without recovering soluble fiber fractions like pectins, gums, and glucans (Grieshop et al., 2001). Crude fiber represents only cellulose, hemicellulose, and lignin (Mertens, 2003). So numerically, TDF > NDF > ADF > crude fiber (Table 1.11). Corn DDGS (30.8-44.1%), SBH (74.6 - 82.1%), and WS (81.8 - 99.8%) all have great variability of TDF concentration, but the variability of NDF in SBH is numerically

less than corn DDGS (28.8 - 44.0%) and WS (69.0 - 83.4%; table 1.1). This result indicates that the soluble fiber fraction in SBH may be more variable. Similarly, corn DDGS (8.6-15.0%), SBH (47.8-51.9%), and WS (51.5-59.7%) all have great variability of ADF concentration, but the variability of lignin content in SBH (1.2 - 4.4%) is numerically less than corn DDGS (1.0 - 8.4%) and WS (5.3 - 9.7%; table 1.1). This indicates that the cellulose concentration in SBH is also more variable than in WS and corn DDGS. The variability of crude fiber of corn DDGS (6.0 - 9.9%) is numerically less than SBH (30.7 - 43.6).and WS (36.6 - 46.2), which reflects the disadvantage of crude fiber procedure- different feed ingredients cover variable fiber fractions. Overall, the NDF measurement is similar to TDF in highly insoluble feed ingredients like corn DDGS and WS; and the greater the soluble fraction of a feed ingredient, the greater difference of TDF and NDF content.

Table 1.11 Fiber comparison of corn distillers dried grains of solubles (DDGS), soybean hulls (SBH), and wheat straw (WS)[1]

Item		Corn DDSG	SBH	WS
TDF, %	Mean, %	34.4	78.9	90.8
	SD, %	3.2	1.9	4.4
	Min, %	30.8	74.6	81.8
	Max, %	44.1	82.1	99.8
NDF, %	Mean, %	34.3	65.5	76.5
	SD, %	4.0	1.8	3.7
	Min, %	28.8	60.9	69.0
	Max, %	44.0	67.7	83.4
ADF, %	Mean, %	11.2	49.6	54.9
	SD, %	2.0	1.2	1.8
	Min, %	8.6	47.8	51.5
	Max, %	15.0	51.9	59.7
Lignin, %	Mean, %	4.3	2.4	7.2
	SD, %	1.9	0.7	1.0
	Min, %	1.0	1.2	5.3
	Max, %	8.4	4.4	9.7
Crude fiber, %	Mean, %	7.9	38.8	41.5
	SD, %	0.9	2.7	2.1
	Min, %	6.0	30.7	36.6
	Max, %	9.9	43.6	46.2

[1]The data of TDF, NDF, and ADF were analyzed in commercial lab based on 16 samples of each feed ingredient. The data of lignin and crude fiber is referenced from Feedipedia (2011, 2014 and 2015).

1.12 Overall summary of literature review

Overall, dietary fiber has been used widely in swine diets. However, a better understanding of how to utilize high fiber ingredients efficiently in commercial swine diets is needed to overcome the challenge of great variability in energy and digestible nutrient content among fiber sources. The composition and physiologic characteristics of dietary fiber are very important to understand the mechanism of how high fiber ingredients depress nutrient utilization due to the variability of fiber composition of each specific fiber source and the unique characterization of each specific fiber source. Developing accurate, rapid, and relatively inexpensive methods to dynamically estimate energy and digestible nutrient content of these high fiber ingredients is necessary to improve the accuracy of diet formulation.

Therefore, the hypotheses of this thesis are: 1) different high fiber feed ingredients and different sources of the same high fiber ingredients have different fermentability; 2) different high fiber feed ingredients affect nutrient utilization in swine differently due to the differences of their effects on gastrointestinal development, specifically cell proliferation and differentiation of the small intestine; 3) modified three-step *in vitro* method might be an effective method for rapid estimation of the digestible and metabolizable energy content and fiber digestibility and fermentability among high fiber feed ingredients.

Chapter 2. Modulation of intestinal cell differentiation in growing pigs is dependent on the fiber source in the diet[1]

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ABSTRACT: Feeding high-fiber diets decreases cost, but also caloric nutritional efficiency by modifying intestinal morphology and function. We analyzed the changes in intestinal cell composition, nutrient transporters and receptors, and cell differentiation induced by fibers from different sources. Forty-six finishing pigs (BW 84 ± 7 kg) were fed 1 of 4 diets: corn-soybean (Control; n = 12), 23% wheat straw (WS; n = 11), 55% corn distillers dried grains with solubles (DDGS; n = 11), and 30% soybean hulls (SBH; n = 12). Pigs were fed 2 meals daily to an amount equivalent to 2.5% of initial BW for 14 d in metabolism cages. Ilea were collected for histological and gene expression analysis after euthanasia. Data were analyzed using Kruskal-Wallis test followed by Dunn's multiple comparisons and differences considered significant when $P < 0.05$. The enterocyte marker was increased ($P < 0.05$) by feeding SBH compared with Control and WS diets. Goblet cells presence was greater ($P < 0.01$) in pigs fed WS and DDGS compared with Control, and in pigs fed WS compared with SBH ($P = 0.02$). *Mucin 2* expression was greater ($P < 0.05$) in pigs fed DDGS and SBH compared with Control. No changes were observed for endocrine and Paneth cells markers, villus and crypt length, or proliferation index. Comapred with the Control, gene expression of receptors for oligopeptides, calcium, glucose, fructose, *free fatty acid receptor 1*, and *G protein-coupled receptors 119* and *84* was increased ($P < 0.05$) by feeding WS and DDGS diets. Feeding SBH diet respressed ($P < 0.05$) by feeding WS and DDGS diets, while DDGS repressed ($P = 0.02$) its expression compared with Control. Pigs fed DDGS had reduced ($P < 0.001$) *fatty acid receptor 2,* .and those fed SBH showed increased ($P < 0.05$) *fatty acid translocase* expression compared with WS and DDGS pigs. Feeding WS and DDGS diets induced ($P < 0.01$) the expression of stem cell marker r-spondin receptor (*LGR5*),

while *olfactomedin 4* was reduced ($P < 0.02$) by feeding DDGS compared with Control. The expression of *delta-like Notch ligand 4* was induced ($P < 0.05$) by all DF compared with Control. Transcription factors *atonal factor 1* and *Wnt family 3A* were suppressed ($P < 0.001$) by WS and DDGS compared with Control. In conclusion, feeding diets containing WS and DDGS modulated intestinal differentiation by promoting goblet cells and altered expression of nutrient receptors and transporters in growing pigs, while feeding SBH had less effect

Key words: dietary fiber, gene expression, goblet cells, intestinal epithelium, nutrient sensing, swine

INTRODUCTION

Ingredients with a high concentration of fiber are commonly used in swine diets to reduce feed cost (Zijlstra and Beltranena, 2013). However, feeding high-fiber diets to pigs results in decreased energy and nutrient utilization and carcass yield (Yin et al., 2000; Noblet and Le Goff, 2001; Chen et al., 2013; Asmus et al., 2014); increased gastrointestinal (**GI**) tract weight (Kass et al., 1980); villus height and crypt size (Jin et al., 1994; Liang et al., 2014); and goblet cell number (Piel et al., 2005). Some researchers have suggested that the GI responds to lower nutrient digestibility of high-fiber diets by increasing absorptive area (Kass et al., 1980; Anugwa et al., 1989). However, models of caloric restriction and reduced nutrient intake have shown reductions in villus height and crypt depth (Genton et al., 2015). Therefore, we hypothesize that reduced nutrient availablility triggers compensatory mechanisms that include changes in cell differentiation to promote protective goblet cells and adaptive response in nutrient sensing and transport.

The functional cells of the small intestine are either absorptive (enterocytes) or secretory (goblet, endocrine, Paneth, tuft, and M-cells). The crypts are populated by stem cells and transient amplifying (**TA**) cells (Barker et al., 2007). Functional cells result from the sequential differentiation of TA cellst thattbecome either absorptive or secretory precursor cells (Karam and Leblond, 1995; Barker et al., 2008). Absorptive precursor cells mature into enterocytes that are responsible for nutrient transport and absorptionwhile secretory precursor cells further differentiate into goblet, Paneth, endocrine or tuft cells (van der Flier and Clevers, 2009). The objective of this study was to investigate the impact of feeding similar amounts of NDF from different fiber sources on GI epithelial proliferation, differentiation, and expression of nutrient receptors and transporters in finishing pigs.

MATERIALS AND METHODS

The animal use protocol was reviewed and approved by the University of Minnesota Institution Animal Care and Use Committee.

Animals and diets

Forty-eight barrows (initial BW 84 ± 7 kg) from Topigs females (Landrace × Yorkshire, Winnipeg, MB) sired by Duroc boars (Compart's Boar Store, Nicollet, MN) were housed individually in metabolism cages (198 cm × 84 cm × 71 cm) at the University of Minnesota Southern Research and Outreach Center (Waseca, MN). The pigs were allotted to provide similar average initial BW among 1 of 4 dietary treatments and provide 12 pigs (replications) per treatment. The control diet contained 7.2% NDF from a typical corn and soybean meal diet. The 3 high fiber diets were formulated to contain a single source of insoluble fiber from 23% wheat straw (**WS**), 55% corn dried

distillers grains with solubles (**DDGS**), and 30% soybean hulls (**SBH**) to achieve approximately 24% NDF content (Table 2.1). All diets were formulated to meet the requirements of pigs of 79 to 90 kg (NRC and National Research Council, 2012).

Pigs were provided feed twice daily (0800 and 1600 h) an amount equivalent to 2.5% of their initial BW. *Ad libitum* access to water was provided from nipple drinkers. The experiment lasted 14-d. Four pigs were excluded from tissue harvest because of sickness or failure to consume the experimental diets (1 pig from the SBH group, 1 out of the DDGS group and 2 pigs from the WS group). On d 15, 44 pigs were weighed, subjected to overnight fasting, and harvested at the Andrew Boss Meat Science Laboratory of the University of Minnesota (St. Paul, MN). Two pieces of ileum, each about 1 cm in length, were collected at the position of about 10 cm proximal to the ileocecal valve. One piece was fixed in formalin and the second piece was snap frozen in liquid nitrogen and stored at -80^{o}C until further processing.

Physicochemical analysis

All diet samples were analyzed by Midwest Laboratories (MWL, Omaha, NE). The AOAC (1995) analysis methods used were as follows: DM (method 930.15), CP (method 992.15), ether extract with acid hydrolysis (method 922.06), total dietary fiber (TDF; method 991.43), and lignin (method 973.18). Determination of ADF (MWL FD 021 procedure) and NDF (MWL FD 022 procedure) was based on the Ankom Technology bag method.

Histological analysis

Ileal samples were fixed in 4% formalin for 24 h, then processed and paraffin embedded following standard histology procedures of the University of Minnesota

Comparative Pathology Shared Resource Laboratory. Villi height and crypt depth were measured in 10 randomly chosen fields at 100 × magnification under light microscopy (Olympus BX53, Center Valley, NJ) on hematoxylin-eosin stained sections. Villus height was defined as the length of a line drawn at the center of the villus from the crypt neck to the tip of the villus. Crypt depth was defined by drawing a line along the center of the crypt from the neck towards the muscularis mucosa up to the point where epithelial cells were observed. Only villi and crypt that had a continuous epithelial cell layer were measured. Mean values of the 10 fields were calculated for each pig.

Goblet cell staining and quantification

Goblet cells of ileum samples were visualized on 4 µm tissue sections stained with periodic acid-Schiff with Alcian blue (PAS-AB, Newcomer Supply, Middleton, WI), with staining procedures followed by the manufacturer's instructions. The stained slides were analyzed at 200 × magnitude under light microscope (Olympus BX53) in 5 randomly chosen fields of mucosa. Within each field, the total area (μm^2) of the mucosa (area delimited by the epithelial surface and the muscularis mucosa) was first measured, and then the area (μm^2) that stained positive for PAS-AB (goblet cell area) was determined using a cell imaging software (CellSense, Olympus, Center Valley, NJ). Data were expressed as the means of the percentage of the total area that was occupied by goblet cells, and were calculated and reported for each pig.

Cell proliferation

Four micron tissue sections were mounted on charged slides, paraffin was dissolved by xylene, and tissues were rehydrated in graded alcohol baths and phosphate-buffered saline. Antigen retrieval was performed by boiling the slides for 30 min in 10 m*M* sodium

citrate buffer (pH = 6.0), slides were subsequently allowed to cool to room temperature before washing 3 times in Tris-buffered saline with 1% Triton (TBS-T) buffer. For immunodetection, a rabbit specific HRP/DAB detection kit (Abcam, Cambridge, MA) was used following manufacturer's instructions. Rabbit monoclonal anti-Ki-67 [SP6] antibody (Biocare Medical Inc., Concord, CA) was used in a 1:200 dilution in TBS-T and incubated for 2 h at room temperature. After secondary antibody incubation and labeling according to instructions, the tissue slides were counterstained with hematoxylin, dehydrated, and a cover slip was placed on slides for further analysis. Ki67 positive cells and total cells per crypt were counted in all well-oriented crypts (10 to 20 crypts per field) found in 10 randomly chosen fields at 200 × magnification under light microscopy (Olympus BX53, Center Valley, NJ). Mean values of the 10 fields of each sample were calculated and reported for each pig.

Gene expression

Total RNA from the ileal samples was isolated using the RNeasy Plus Universal Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Total RNA was quantified using a NanoDrop 2000 instrument (Thermo Scientific, Wilmington, DE), and 1000 ng of RNA were reverse transcribed using the High Capacity cDNA reverse Transcription Kit (Applied Biosystems, Foster City, CA). The expression of genes of interest was determined using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a StepOne-Plus system (Applied Biosystems, Foster City, CA). The PCR conditions were: initial activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, denaturation, and annealing at 60°C for 60 s. The primer sequences used are shown in Table 2.2.

Data analysis

Relative gene expression was calculated using the primer efficiency values as described by Pfaffl (2001), and Ct values > 38 were considered non-detectable. The specific epithelial markers, *EPCAM* and *VIL1* (Madison et al., 2002; El Marjou et al., 2004; Li et al., 2007) along with the housekeeping genes *GAPDH* and *18s*, were used as reference genes, and the target gene expression of each sample was normalized to the mean of the control group. All data were evaluated for normality using D'Agostino and Pearson tests. Normalized gene expression levels were analyzed using ANOVA with Kruskal-Wallis test, followed by Dunn's multiple comparisons test using GraphPad 7.0 (GraphPad Software, Inc., La Jolla, CA). The values for Ki67 positive cells per crypt, goblet positive area per mucosa area (%), and relative gene expression were tested for normality, using the pig as the experimental unit, and analyzed using ANOVA with Kruskal-Wallis test followed by Dunn's multiple comparisons test.

RESULTS

Effects of fiber sources on differentiated intestinal cells

There were no differences in villus height or crypt depth among control, WS, DDGS, or SBH dietary treatments (Table 2.3).

Absorptive cells: The presence of enterocytes, as measured by the expression of *FABP2*, was not different among the control, WS, or DDGS diets (Figure 2.1A). However, expression of *FABP2* in pigs fed the SBH diet was greater ($P < 0.05$) than in pigs fed the control and WS diets. Pigs fed the SBH diet tended to have greater ($P = 0.07$) *FABP2* expression compared with those fed the DDGS diet.

The expression of the basic amino acid receptor *GRPC6A* and the oligopeptide transporter *PEPT1* were increased ($P < 0.05$) by feeding the high fiber diets compared with the control diet (Table 2.4). Expression of the glucose transporter *GLUT2* and the fructose transporter *GLUT5* were increased by feeding the WS ($P < 0.05$) and DDGS ($P < 0.001$) diets compared with the control diet, and were not affected by feeding the SBH ($P > 0.99$) diet. All tested free fatty acid receptors were differentially regulated by diet. Expression of FFAR1 was greatest in pigs fed the DDGS ($P < 0.001$ compared with Control) and WS ($P < 0.001$ compared with Control) diets, than for pigs fed SBH ($P = 0.4$ compared with Control or $P < 0.01$ compared with WS and DDGS). Receptor *FFAR4* expression was regulated in a similar way to *FFAR1*, but its expression in pigs fed the DDGS diet tended ($P = 0.08$) to be greater than for those fed the SBH diet. The regulation of the expression of *GPR119* was similar, but the levels of expression induced by WS and DDGS diets were ten times greater than the changes in *FFAR1* expression (2 and above 28 fold for *FFAR1* and *GPR119* respectively). The *GPR84* medium chain fatty acid receptor expression was also increased by feeding the WS and DDGS diets ($P < 0.05$) compared with the pigs fed the control diet, but feeding the SBH diet had no effect ($P < 0.99$). In contrast, the expression of *FFAR2* was induced by feeding the SBH diet compared with pigs consuming the WS ($P = 0.006$) and DDGS ($P < 0.0001$) diets, and was repressed by feeding DDGS ($P = 0.02$) compared with control diet. The *CD36* fatty acid translocase expression was also increased in pigs fed the SBH diet compared with those fed WS ($P = 0.02$) and DDGS ($P = 0.0001$) diets, but was not different compared with those fed the control diet ($P = 0.16$). The expression of the calcium sensing receptor (*CASR*) was increased by feeding WS ($P < 0.005$) and DDGS ($P < 0.02$) diets compared

with control and SBH diets, while the expression of the receptor for sweet and umami taste (*TAS1R3*) was induced by feeding the SBH diet compared with WS ($P = 0.003$) and DDGS ($P < 0.0001$) diets, and was repressed in pigs fed the DDGS diet compared with those fed the control ($P = 0.02$).

Secretory cells: Presence of goblet cells was assessed by histology (Figure 2.1B and 2.1C) and expression of *MUC2* (Figure 2.1 D). After measuring similar mucosal areas in the tissue samples, feeding WS ($P < 0.001$) and DDGS ($P = 0.01$) diets increased the goblet cell area (% of mucosa) compared with the control diet, while pigs fed the SBH diet had less goblet cell area than the pigs fed WS ($P = 0.02$). Feeding the DDGS and SBH diets induced greater *MUC2* expression ($P < 0.05$) compared with the control diet, but no differences were found among the DDGS, SBH, and WS treatments.

Presence of Paneth cells was evaluated by *LYZ1* expression. There were no differences among control and the high fiber dietary treatments. Pigs fed the WS diet expressed more *LYZ1* ($P = 0.01$) than pigs fed SBH (Figure 2.1 E). Using *CHGA* as an endocrine cell marker, pigs fed SBH tended ($P = 0.054$) to have less *CHGA* expression compared with pigs fed the control diet. Among the high fiber diets, feeding SBH repressed *CHGA* ($P = 0.0001$) expression compared with pigs fed DDGS (Figure 2.1F).

Effects of fiber sources on cell proliferation

Proliferating Ki-67 positive cells per crypt were counted in all ileal samples (Figure 2.2A). There were no differences in the number of proliferating cells per crypt for pigs fed the control diet (12.39 ± 2.3 % cells/crypt) compared with those fed WS (11.52 ± 33.9 % cells/crypt), DDGS (13.25 ± 2.8 % cells/crypt), and SBH (9.9 ± 4.3 % cells/crypt) diets. Furthermore, there were no differences in proliferating cells per crypt among

DDGS, SBH, and WS dietary treatments (Figure 2.2B). These observations indicate that after a 14-d feeding period, fiber source did not have major effects on intestinal cell proliferation.

Effects of fiber sources on cell differentiation signaling

Intestinal stem cell activation was determined by the expression levels of *LGR5* (Barker et al., 2007; Gonzalez et al., 2013; Koo and Clevers, 2014) and *OLFM4* (Farin et al., 2012; Clevers, 2013a; Gonzalez et al., 2013; Guezguez et al., 2014). Compared with the control diet, feeding the WS ($P = 0.001$) and DDGS ($P < 0.0001$) diets increased *LGR5* expression, but there was no difference in the levels induced by feeding the SBH diet (Figure 2.3A). Among fiber sources, feeding DDGS increased *LGR5* expression compared with feeding SBH ($P = 0.017$). The expression of *OLFM4* was repressed ($P < 0.05$) in pigs fed the DDGS diet compared with those fed the control diet, and the level of expression in the DDGS ($P = 0.02$) treatment was less than the expression observed in when feeding the SBH diet (Figure 2.3B).

The signals that modulate transient amplifying (TA) cell differentiation were evaluated using *HES1*, *DLL4*, *ATOH1*, and *SOX9* expressions (Shroyer et al., 2005; Pin et al., 2012; Barker, 2013; Sakar et al., 2014; Shimizu et al., 2014; Cao et al., 2015). The expression of *HES1* mediates the transition of TA cells to absorptive precursors and enterocytes. However, no differences of *HES1* expression were observed among the dietary treatments (Figure 2.3C). The product of *ATOH1* induces the commitment of the TA cells to the secretory lineage, and its action is blocked by the presence of HES1. Compared with the control diet, feeding WS ($P = 0.004$) and DDGS ($P = 0.0002$) diets repressed the expression of *ATOH1*, but feeding SBH ($P < 0.99$) had no effect (Figure

2.3D). We also analyzed the expression of *SOX9*, a factor which expression is associated with the secretory lineage differentiation toward Paneth and tuft cells (Mori-Akiyama et al., 2007; Huch and Clevers, 2011; Pin et al., 2012; Gonzalez et al., 2013). Compared with the control diet (1.0 ± 0.02), feeding the WS (0.72 ± 0.09, $P < 0.0001$) and DDGS (0.81 ± 0.04, $P = 0.0001$) diets reduced *SOX9* expression, and there was no difference between feeding the control and SBH diets (0.92 ± 0.06, $P = 0.16$). In addition, there were no differences in *SOX9* expression among DDGS, SBH, and WS dietary treatments.

The intestinal stem cell niche is regulated mostly by Notch and Wnt signaling (de Santa Barbara et al., 2003; Pin et al., 2012; Barker, 2013; Gonzalez et al., 2013; Sakar et al., 2014; Tian et al., 2015). To determine the major changes in Wnt and Notch signaling pathways, we analyzed the expression of the Wnt ligand *WNT3A*, and the Notch ligand *DLL4*, which restricts the commitment of TA cells to a secretory fate. Pigs fed both the WS ($P = 0.0001$) and DDGS ($P < 0.0001$) diets had reduced *WNT3A* expression compared with those fed the control diet (Figure 2.3E). Among fiber sources, the expression of *WNT3A* for pigs fed DDGS ($P = 0.0036$) was less than those fed SBH (Figure 2.3E). The expression of *DLL4* was increased when feeding the DDGS ($P = 0.003$), SBH ($P = 0.034$), and WS ($P < 0.0001$) diets compared with feeding the control diet, but there were no differences among the 3 high fiber diets (Figure 2.3F).

DISCUSSION

After histological analysis of the ileal samples of pigs fed the experimental diets for 14-d, we did not observe changes in villus height, crypt depth, or epithelial cell proliferation. Pin et al. (2012) developed a mathematical model using data from rodent studies, and determined that at least 14-d are required to observe significant changes in

the intestinal crypt structure after a dietary insult or modification. The lack of changes in the intestinal architecture and proliferation rates that we observed are in agreement with this estimate of a minimum 14-d of feeding high fiber diets, because studies have shown that changes in intestinal architecture occur when feeding high fiber diets for 25 to 30 d (Gutiérrez et al., 2002; Chen et al., 2015). However, we observed changes in nutrient receptors and transporters, characterized by an increase in the number of goblet cells, increased *MUC2* expression, and no differences in Paneth (*LYZ1*) and endocrine cells (*CHGA*) when feeding these high fiber diets, compared with feeding the control diet.

Although the pig has been recognized as a good model for the study of chemosensory molecules in humans (van der Wielen et al., 2014; Kim et al., 2016; Roura et al., 2016), little is known about the expression and dietary regulation of nutrient receptors and sensing molecules in the pig. To our knowledge, this is the first study to report changes of these molecules in the pig ileum induced by different fiber sources. The peptide receptors, *GPRC6A* and *PEPT1*, along with the *CASR*, participate in regulating intestinal motility, fluid absorption (Conigrave et al., 2007; Tang et al., 2016); intestinal growth and differentiation mediated by amino acids (MacLeod, 2013; Mine and Zhang, 2015; O'Brien and Corpe, 2016); regulation of immune response and barrier function in piglets (Boudry et al., 2014; Huang et al., 2016)(Boudry et al., 2014; Huang et al., 2016). In addition, they are involved in regulating hormone secretion, especially glucagon-like peptide 1 (GLP-1) and glucose insulinotropic peptide (GIP) (Reimann et al., 2012; Diakogiannaki et al., 2013). However, little is known regarding the factors that affect their expression in the intestine. In pigs, the inclusion of phytase in the diet has been shown to induce *PEPT1* expression (Vigors et al., 2014). Chitosan oligosaccharides have

been shown to increase *CASR* expression in piglets challenged with bacterial polysaccharide, resulting in an anti-inflammatory response (Huang et al., 2016). In our study, the DDGS diet contained the greatest concentration of CP, followed by the SBH and WS diets, which may explain the stronger effect of the DDGS diet on inducing the expression of *PEPT1* and *CASR*. The expression of the glucose (*GLUT2*) and fructose (*GLUT5*) transporters induced by feeding the WS and DDGS diets was likely due to the addition of corn starch to these diets. However, we were not expecting GLUT5 expression to change, because changes in its expression have been associated with feeding pigs diets that contain more than 50% of carbohydrates, and these changes are restricted to the proximal small intestine (Moran et al., 2010). Another unexpected change observed in this study was the reduction of expression of the umami taste receptor, *TAS1R3*, induced by feeding the DDGS diet in comparison with the strong induction by feeding the SBH diet. The *TAS1R3* receptor is important for the recognition of sugars, and has been suggested to participate in metabolic regulation (Toyono et al., 2003; Gribble, 2012; Silva et al., 2014; Xiao et al., 2014). However, its regulation in the ileum is not well defined (Kim et al., 2016). The greater effects we observed in nutrient sensing and transport receptors in our study were those of fatty acid receptors, and the transporter CD36. These changes were likely due to the release of short chain fatty acids resulting from microbial fermentation in the distal intestine (Zhang et al., 2014; Macia et al., 2015). Further research is needed to understand the mechanisms involved in these changes.

As previously discussed, the increase in number of goblet cells induced by feeding high fiber diets is well-documented in rats (Tanabe et al., 2007; Kanauchi et al., 2008; Ito

et al., 2009) and pigs (Piel et al., 2005; Hedemann et al., 2006). However, we observed a clear difference in the magnitude of this effect due to the type of fiber source fed. This observation supports the suggestion from other studies that the effects of fiber on intestinal morphology are dependent on the fiber characteristics (e.g. fermentability, solubility, viscosity, water holding capacity), but are also affected by the protein and lipid fractions of the fiber source (Hedemann et al., 2006; Serena et al., 2008; Ito et al., 2009; Lindberg, 2014). The interactive effects of protein and lipids with fiber in the GI tract is of particular importance when considering the implications of using purified (e.g. cellulose) versus complex fiber sources (e.g. whole grains and DDGS) for research purposes.

Unfortunately, published information on how complex, high fiber diets modulate intestinal cell differentiation or proliferation is very limited, and more research has been devoted to studying dietary fat compared with protein and fiber. A recent report showed that feeding a high fat diet to rats resulted in impaired endocrine cell differentiation that favored enterocyte differentiation via the modulation of the expression of factors like Math1, neurogenin 3 and neuro D1 (Mah et al., 2014). In a previous study, we observed an increased number of endocrine cells in the mouse stomach induced by dietary lipid (Saqui-Salces et al., 2012), but no information is available on the possible role of proteins and the complex chemical composition of various ingredient sources, such as those used in the current study, on GI cell differentiation. Therefore, further research is necessary to determine if complex, non-purified fiber and lipid sources (i.e. commodity feed ingredients) elicit the same effects on GI physiology as observed in rodent models fed purified sources of fiber and lipids.

The observed increase in the presence of goblet cells induced by fiber in WS and DDGS diets, without major changes in enterocyte markers, may reflect a net decrease in absorptive area. In contrast, the responses observed when feeding the SBH diet showed no net increase in goblet cell area, but an increase on *FABP2* expression. These differences are likely due to the fact that intestinal stem cells, located at the base of the crypt, divide asymmetrically to maintain the stem cell population, and produce differentiated cell types that rise from the transient amplifying compartment in the crypt (Clevers, 2013a; Clevers, 2013b; Middendorp et al., 2014). Due to lateral inhibition, the commitment of TA cells to differentiate into absorptive enterocytes or to secretory cells (goblet, endocrine and tuft cells) is mutually exclusive (Mori-Akiyama et al., 2007; Gerbe et al., 2011; VanDussen et al., 2012; Petersen et al., 2014). Therefore, the expansion of the secretory lineage (i.e. goblet, Paneth, endocrine and tuft cells) occurs at the expense of the absorptive lineage (i.e. enterocytes). Considering these differentiation mechanisms, in order for the intestine to gain absorptive capacity, an increase in the overall number of enterocytes and/or an increase in enterocyte function are needed. In our study, feeding the WS and DDGS diets seemed to favor the secretory lineage that would lead to a net loss of absorptive capacity, while feeding the SBH diet resulted in responses comparable with feeding the control diet, except for the *MUC2* expression. It appears that a longer feeding time for these diets is necessary to determine if feeding a SBH diet would eventually induce a greater number of goblet cells.

To understand how the different fiber sources modulate intestinal cell differentiation, we analyzed the gene expression of molecules related to stem cell activation and function, *LGR5* and *OLFM4*. The increase in *LGR5* expression was induced by feeding

the WS and DDGS diets compared with feeding the control and SBH diets, but the expression of *OLFM4* was reduced. These results suggest that feeding the WS and DDGS diets may promote Notch, but not Wnt signaling pathways (Guezguez et al., 2014; Liu et al., 2016). This observation is further supported by the strong repression of *WNT3a* expression that occurred by feeding WS and DDGS, but not by feeding SBH, while the Notch ligand *DLL4* was induced by feeding all of the high fiber diets compared with the control. The expression of the transcription factor *HES1*, which is the Notch signaling factor associated with the inhibition of the secretory program, and thus, favoring the enterocyte absorptive lineage (Suzuki et al., 2005; Barker et al., 2008), was not affected by feeding the high fiber diets. The expression of *ATOH1*, a factor that drives the differentiation towards the intestinal secretory lineage (Gerbe et al., 2011; VanDussen et al., 2012), was decreased when pigs were fed the WS and DDGS diets. Although the presence of HES1 blocks ATOH1, and thus, favors enterocyte differentiation, it was unclear from analysis of our data whether the promotion of goblet cells was induced by dietary fiber at this first stage of the cell differentiation process. We expected an increase on *HES1* with or without changes in *ATOH1* expression. We also observed a decrease in *SOX9* expression resulting from feeding the WS and DDGS diets compared with the control diet, which suggests that WS and DDGS diets increase the presence of goblet cells. The changes on other secretory cells (i.e. Paneth, endocrine and tuft cells) were not apparent in our study, which was probably due to the short exposure time to the diets (14-d). The median life span of these cell types is greater than 30 d, and longer feeding periods of high fiber diets are required to study changes on those cell populations. Other important factors (bone morphogenetic proteins, hedgehog ligands, CDX1, etc.)

participating in these differentiation processes have been identified in rodent models. However, only some of those factors, namely *LGR5*, *OLFM4*, *SOX9*, transforming growth factor alpha and epithelial growth factor, have been evaluated in the pig intestine (Gonzalez et al., 2013). As a result, we were unsuccessful in designing primer sequences that would recognize the corresponding sequences in the pig intestine that could be identified by homology in a specific and quantitative manner. It is unknown whether the intestinal cell differentiation in the pig occurs exactly as it does in the mouse, and this needs to be confirmed with further research.

CONCLUSION

Overall, results from our study show that dietary inclusion of fiber favors the intestinal differentiation pathways that lead to goblet cells, and induces significant changes in nutrient receptors which may attempt to compensate for the reduced nutrient digestibility in the presence of fiber. Furthermore, the physiological effects of fiber in pig diets is dependent of the fiber type and source, with WS and DDGS being more detrimental than SBH for increasing the presence of goblet cells, and these effects appear to be the result of fiber reducing Wnt signaling.

Table 2.1. Ingredient composition and nutrient content of diets containing wheat straw (WS), corn distillers dried grains with solubles (DDGS), and soybean hulls (SBH)

Item	Control	WS	DDGS	SBH
Ingredient (%)				
Corn, yellow dent	79.79	-	-	-
Soybean meal	17.86	-	-	-
Wheat straw (WS)	-	23.00	-	-
Corn distillers dried grains with solubles (DDGS)	-	-	55.00	-
Soybean hulls (SBH)	-	-	-	30.00
Spray dried porcine plasma	-	4.73	4.73	4.73
Corn starch	-	61.10	34.73	56.66
Casein	-	3.00	3.00	3.00
Fish meal, menhaden	-	6.74	-	3.77
Titanium dioxide	0.05	0.50	0.50	0.50
Dicalcium phosphate, 18.5% P	0.65	-	-	0.30
Limestone	0.92	0.32	1.44	0.44
Sodium chloride	0.30	0.30	0.30	0.30
Grow-finish vitamin and mineral premix[1]	0.25	0.30	0.30	0.30
Total	100	100	100	100
Analyzed nutrient composition (%), DM basis				
GE, kcal/kg	4340	4167	4475	4103
CP	17.60	13.00	23.07	13.33
Acid hydrolyzed ether extract	2.66	2.87	6.20	2.40
ADF	2.60	12.63	8.77	14.63
NDF	8.50	24.20	19.57	21.53
Titanium, %	0.40	0.32	0.37	0.29

[1]The vitamin and trace mineral premix (ANS Swine G-F premix) provided the following (per kg of diet): vitamin A, 3,527,392 I.U.; vitamin D_3, 661,386 I.U.; vitamin E as dl-alpha tocopherol acetate, 13,228 I.U.; vitamin K (MPB), 1,323 mg; riboflavin, 2,205 mg; niacin, 13,228 mg; pantothenic acid, 8,818 mg; vitamin B_{12}, 13 mg; iodine (EDDI), 119 mg; selenium (Na selenite), 119 mg; SQM organic zinc, 22,046 mg; SQM organic iron, 13,228 mg; SQM organic manganese, 454 mg; SQM organic copper, 1,543 mg.

Table 2.2. Genes of interest and primer sequences used in this study

Gene[1]	Forward Sequence	Reverse Sequence
18s	TGGAGCGATTTGTCTGGTTA	ACGCTGAGCCAGTCAGTGTA
ATOH1	CACGGGCTGAACCACGCCTT	GGTACCCGCGCTTGCTTCGT
CASR	CCCCTCACTGTTGTGCTCCC	CTTGCGTCTGTCTCATCGCTGTA
CD36	AGGAATCCCACTGCCTCACT	TTGCTTCAAGTGCTGGGTCA
CHGA	AAGGAGATGCAGAGGGGTTG	AAAGGGGACAACAGAGCCAG
DLL4	TCATCATCGAAGCTTGGCAC	GCGCTTCTTGCATAGACGTG
EPCAM	GCGGCCCAACAAGGATGTGT	CCCAGCCTTTGACCCAGTCA
FABP2	CCGGCAAATACCAAGTACAGA	GCCCCTTCTCCCCAGTCAGGGTCTCC
FFAR1	TCACGGCCTTCTGCTATGTG	CCCTTAGCTTCCGTCTGTGG
FFAR2	CTGCCTGGGATCGTCTGTG	CATACCCTCGGCCTTCTGG
GAPDH	ATCCTGGGCTACACTGAGGAC	AAGTGGTCGTTGAGGGCAATG
GLUT2	TTTTGGGTGTTCCGCTGGAT	GAGGCTAGCAGATGCCGTAG
GLUT5	TGTGTGGCTCCTGGTAACAC	TCGGCCATGTTCGATTCCTT
GPR119	CAGCTTCTTCGCCGTGTTTC	GTGCTCTGTCTTGCGGATCT
GPR84	CAGCTTTGACCGCATTCGAG	CCATTGAGCCAGGTGAGGTT
GPRC6A	GCCGGGATTTGTCCACAGTA	TGGTTGAAAGGCATTGGGGT
HES1	TGTCAACACGACACCGGATA	TCCAGAATGTCCGCCTTCTC
LGR5	CCTTGGCCCTGAACAAAATA	ATTTCTTTCCCAGGGAGTGG
LYZ1	GGTCTATGATCGGTGCGAGT	AACTGCTTTGGGTGTCTTGC
MUC2	GGCTGCTCATTGAGAGGAGT	ATGTTCCCGAACTCCAAGG
OLFM4	GTCAGCAAACCGGCTATTGT	TGCCTTGGCCATAGGAAATA
PEPT1	TTGTGGCTCTGTGCTACCTG	TCCGTTGTGGTCGAAGTCTG
SOX9	GCAAGAATAAGCCGCACGTC	CTTGAAGATGGCGTTGGGAG
TAS1R3	GCTGGGCGACAGGACAG	TTGATTTCCTCCACAGCCAT
VIL1	CACCATGACCAAACTGAACG	TCGAAGAAGCTGCCATAGGT
WNT3A	GGTCACGTGTACCGAAGGAT	GCGACTTCCTCAAGGACAAG

[1]Names of genes: *18s*: ribosomal 18S subunit, *ATOH1*: atonal homolog 1, *CASR*: calcium sensor receptor, *CD36*: fatty acid translocase, *CHGA*: chromogranin A, *DLL4*: delta-like ligand 4, *EPCAM*: epithelial cell adhesion molecule, *FABP2*: fatty acid binding protein 2, *FFAR1*: free fatty acid receptor 1 (also known as G protein-coupled receptor 40), *FFAR2*: free fatty acid receptor 2 (also known as G protein-coupled receptor 43), *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase, *GLUT2*: solute carrier family 2 member 2 (SLC2A2), *GLUT5*: solute carrier family 2 member 5 (SLC2A5), *GPCR6CA*: G protein-coupled receptor class C, group 6 member A, *GPR119*: G protein-couple receptor 119, *GPR84*: G protein-coupled receptor 84, *HES1*: hairy and enhancer of split-1, *LGR5*: leucine rich repeat containing G protein-coupled receptor 5, *LYZ1*: lysozyme 1, *MUC2*: mucin 2, *OFLM4*: olfactomedin 4, *PEPT1*: solute carrier family 15 member 1 (*SLC15A1*), *SOX9*: sex determining region Y-box 9, *TAS1R3*: taste 1 receptor member 3 (umami receptor), *VIL1*: villin 1, *WNT3A: wingless-type* (Wnt) MMTV integration site family 3A.

Table 2.3. Villus height and crypt depth in the ileum of pigs fed a corn and soybean meal diet (Control), wheat straw (WS), corn distillers dried grains with solubles (DDGS), and soybean hulls as the primary source of dietary fiber.

	Control	**WS**	**DDGS**	**SBH**	***P* - value**
Villus height[1], µm	345.5 ± 8.6	360.1 ± 24.1	368.1 ± 26.1	364.9 ± 20.2	0.16
Crypt depth[1], µm	144.6 ± 24.8	147.5 ± 25.4	168.9 ± 37.9	131.7 ± 26.4	0.51

[1]Values are means ± SD of $n = 12$ for Control, $n = 10$ for WS, $n = 10$ for DDGS, and $n = 11$ for SBH.

Table 2.4. Relative gene expression of nutrient sensors and transporters in the ileum of pigs fed a corn and soybean meal diet (Control), wheat straw (WS), corn distillers dried grains with solubles (DDGS), and soybean hulls as the primary source of dietary fiber.

Gene[1]	Control	WS	DDGS	SBH	*P* - value
Amino acids, oligopeptides and related receptors					
GPRC6A[2]	1.0 ± 0.07^{x}	1.61 ± 0.26^{y}	1.42 ± 0.22^{y}	1.42 ± 0.16^{y}	0.0002
PEPT1[3]	1.0 ± 0.09^{x}	2.44 ± 0.64^{y}	2.39 ± 0.62^{y}	1.68 ± 0.35^{y}	<0.0001
CASR[4]	1.0 ± 0.10^{x}	3.13 ± 0.89^{y}	3.07 ± 0.90^{y}	1.05 ± 0.24^{x}	<0.0001
Sugars					
GLUT2[5]	1.0 ± 0.08^{x}	1.67 ± 0.44^{yz}	1.72 ± 0.36^{z}	1.12 ± 0.11^{xy}	<0.0001
GLUT5[6]	1.0 ± 0.16^{x}	1.84 ± 0.52^{y}	1.99 ± 0.45^{y}	1.04 ± 0.19^{x}	<0.0001
TAS1R[7]	1.0 ± 0.31^{yz}	0.69 ± 0.41^{xy}	0.26 ± 0.10^{x}	5.67 ± 4.29^{z}	<0.0001
Fatty acids					
FFAR1[8]	1.0 ± 0.08^{x}	2.28 ± 0.48^{yz}	2.42 ± 0.37^{z}	1.14 ± 0.10^{xy}	<0.0001
FFAR2[9]	1.0 ± 0.25^{yz}	0.82 ± 0.23^{xy}	0.56 ± 0.14^{x}	2.00 ± 0.46^{z}	<0.0001
FFAR4[10]	1.0 ± 0.05^{x}	2.37 ± 0.51^{z}	2.28 ± 0.45^{yz}	1.34 ± 0.52^{xy}	<0.0001
GPR119[11]	1.0 ± 0.29^{x}	28.48 ± 17.89^{y}	44.2 ± 19.47^{y}	0.72 ± 0.31^{x}	<0.0001
GPR84[12]	1.0 ± 0.12^{x}	2.70 ± 0.88^{y}	2.97 ± 0.38^{y}	0.98 ± 0.12^{x}	<0.0001
CD36[13]	1.0 ± 0.16^{xy}	0.94 ± 0.16^{x}	0.82 ± 0.36^{x}	1.40 ± 0.26^{y}	0.0003

[x, y, z] Different letters indicate significant differences ($P \leq 0.05$)

[1]Values are means ± SD

[2]*GPCR6CA:* G protein-coupled receptor class C, group 6 member A

[3]*PEPT1*: peptide transporter solute carrier family 15 member 1 (*SLC15A1)*

[4]*CASR*: calcium sensor receptor

[5]*GLUT2*: solute carrier family 2 member 2 (*SLC2A2)*

[6]*GLUT5*: solute carrier family 2 member 5 (*SLC2A5*)

[7]*TAS1R3*: taste 1 receptor member 3 (sweet and umami receptor

[8]*FFAR1*: free fatty acid receptor 1

[9]*FFAR2*: free fatty acid receptor 2

[10]*FFAR4*: free fatty acid receptor 4

[11]*GPR119*: G protein-couple receptor 119

[12]*GPR84*: G protein-coupled receptor 84

[13]*CD36*: fatty acid translocase

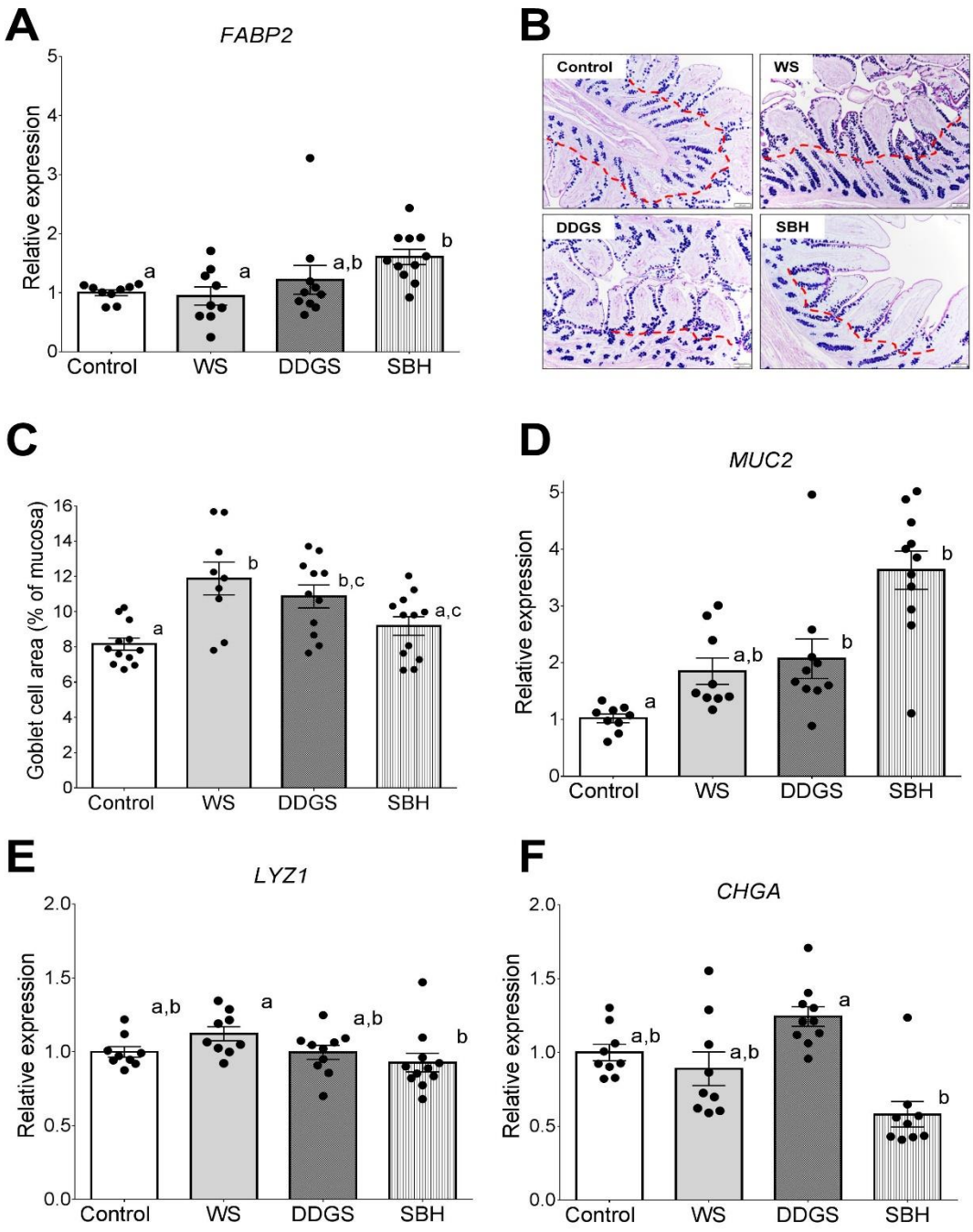

Figure 2.1. Relative gene expression of the enterocyte marker *FABP2 (A)*. Representative images of ileal sections stained with PAS/Alcian blue to identify goblet cells (B) and quantification of the area occupied by goblet cells in the mucosa (C). Relative gene expression of mucin 2 (*MUC2)* (D), lyzosime 1 (*LYZ1)* (E) and chromogranin A (*CHGA)* (F) in the ileum of growing pigs fed Control, wheat straw (WS), corn DDGS (DDGS) and soybean-hull (SBH) diets for 14 d. Different letters indicate significant differences ($P \leq 0.05$). Bars represent mean ± SEM.

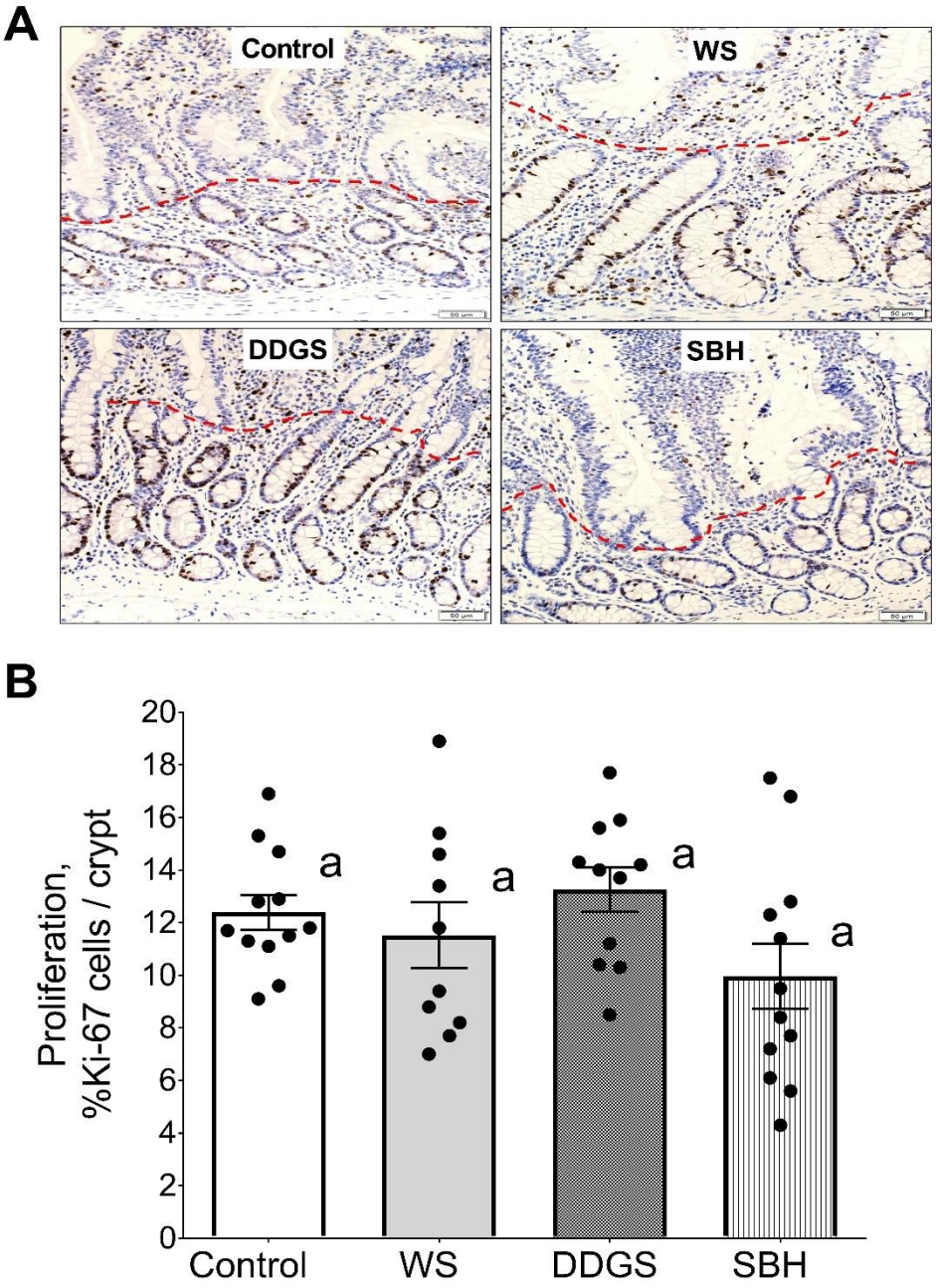

Figure 2.2. (A) Representative images of ileal sections immunostained for the proliferation marker Ki-67 (brown nuclei). Red dotted lines indicate the crypt neck. (B) Quantification of proliferation expressed as the percentage of Ki-67 cells / crypt. Different letters indicate significant differences ($P \leq 0.05$). Bars represent means ± SEM.

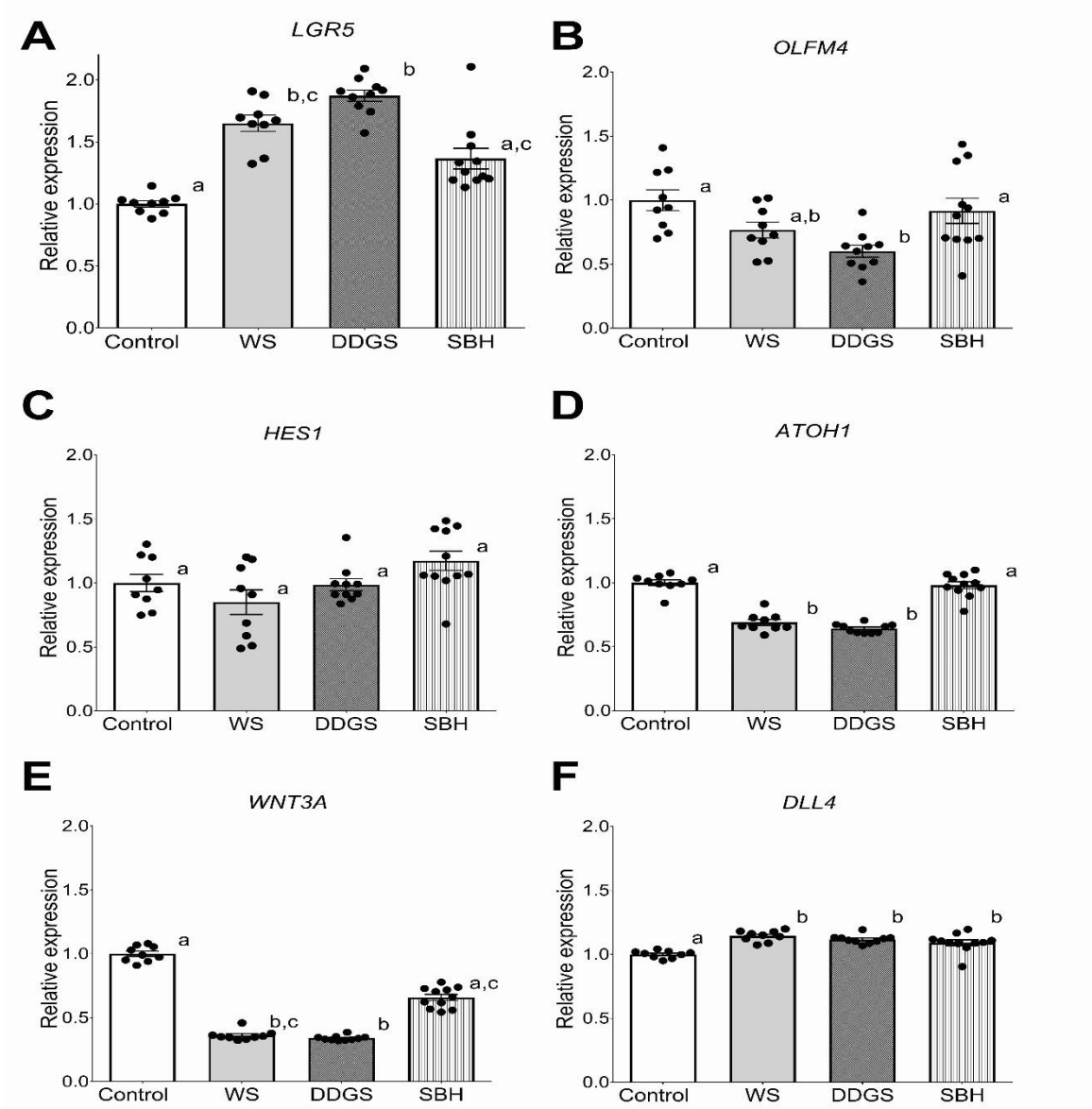

Figure 2.3. Relative gene expression of leucine rich repeat containing G-protein-coupled receptor 5 (*LGR5,* A), olfactomedin 4 *(OLFM4*, B), hairy and enhancer of split 1 (*HES1*, C), atonal transcription factor 1 (*ATOH1*, D), wingless-type MMTV integration site family 3A (*WNT3A*, E) and delta-like Notch ligand 4 (*DLL4*, F) in the ileum of growing pigs fed Control, wheat straw (WS), corn distillers dried grains with solubles (DDGS) and soybean-hull (SBH) diets for 14 d. Different letters indicate significant differences ($P \leq 0.05$). Bars represent means ± SEM.

Chapter 3. Differences of *in vitro* hydrolysis, fermentation, and estimated energy among and within high fiber ingredients using a modified three-step procedure in growing pigs[1]

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ABSTRACT: Measurement of *in vitro* dry matter digestibility (IVDMD) and gas production can be used to rapidly estimate apparent total tract digestibility of DM and GE in feed ingredients used in swine diets. The objectives of this study were to measure IVDMD of feed ingredients with high fiber content, compare *in vitro* gas production kinetics, and estimate ME contributions resulting from fiber fermentation. Wheat straw (WS; 16 sources; NDF 69.0-83.4%), soybean hulls (SBH; 16 sources; NDF 60.9-67.7%) and corn dried distillers' grains with solubles (DDGS; 16 sources; NDF 28.8-44.0%) were evaluated. Each 2 g sample was hydrolyzed for 2 h with pepsin and for a subsequent 4 h with pancreatin. Hydrolyzed residues were filtered, washed, dried, weighed, pooled within the same sample and used for subsequent fermentation using swine fecal inocula. The volume of gas produced was recorded at 11 time points within 72 h of incubation. Gas production kinetics were fitted by a non-linear model and parameters were analyzed using a mixed model. The IVDMD from enzymatic hydrolysis ($IVDMD_h$) in corn DDGS (55.7%) was greater ($P < 0.05$) than SBH (19.7%), which was greater ($P < 0.05$) than WS (14.5%). Large intestine fermentation ($IVDMD_f$) of SBH (68.5%) was greater ($P < 0.05$) than corn DDGS (52.7%), which was greater than WS (41.8%). Total tract digestibility ($IVDMD_t$) was greatest ($P < 0.01$) for corn DDGS (79.2%) followed by SBH (74.8%), which were greater than WS (50.2%). The asymptotic gas production (A, mL/g substrate) was greater ($P < 0.05$) for SBH (293) than corn DDGS (208), and was greater than WS (53). There were differences ($P < 0.01$) of $IVDMD_h$ among sources of WS, SBH, and corn DDGS, while $IVDMD_f$ and $IVDMD_t$ were different ($P < 0.01$) among sources of SBH, but not among sources of corn DDGS or WS. There were no differences in

asymptotic gas production among sources of WS, SBH, or corn DDGS. In conclusion, the modified three-step procedure was useful for detecting the variability of DM digestibility among and between WS, SBH, and corn DDGS sources.

Key words: corn dried distillers' grains with solubles, *in vitro* dry matter digestibility, metabolizable energy, soybean hulls, wheat straw

INTRODUCTION

High fiber ingredients are used in swine diets because of the cost competitiveness and relative abundance compared with corn and soybean meal (Zijlstra and Beltranena, 2013), but these ingredients have variable ME content. Therefore, dynamic prediction equations are needed for individual ingredients to increase precision of prediction. The ME content of corn distillers dried grains with solubles (**DDGS**) varies from 3,153 to 4,336 kcal/kg (Pedersen et al., 2007; Graham et al., 2014a). Many published ME prediction equations include fiber content, as measured by NDF or total dietary fiber (**TDF**), as a necessary input that increases precision of ME estimates (Kerr et al. 2013). A measure of fiber is included in DDGS prediction equations because there is considerable variability in total dietary fiber (**TDF**; 28.6 to 34.9%) content as well as differences in apparent total tract digestibility (**ATTD**; 29.3-57%; Urriola et al., 2010) among sources. Therefore, further improvement in precision of ME predictions can increase using estimates of TDF content and ATTD from estimates of hindgut fermentation (Anguita et al., 2006; NRC, 2012).

A modified three-step *in vitro* procedure that involves pepsin and subsequent pancreatin hydrolysis followed by fecal fermentation has been developed and used to

measure *in vitro* dry matter digestibility (**IVDMD**) and total gas production of various feed ingredients for swine (Bindelle et al., 2007; Jha et al., 2015). *In vitro* fermentation and gas production has been compared among feed ingredients with variable concentrations of soluble dietary fiber (**SDF**), but there are no data that compare the effectiveness of this technique among sources with high insoluble dietary fiber (**IDF**) content. Insoluble dietary fiber tends to be less fermentable than ingredients high in SDF content. Also, this technique has not been used for ME estimation, or to evaluate the variability of gas production resulting from fermentation with different fiber sources, including corn DDGS. The hypothesis of the present study was that different high fiber ingredients, and different sources within each ingredient, have different *in vitro* enzymatic hydrolysis, fermentation characteristics, and contribution to energy from fermentation.

Therefore, the objective was to measure *in vitro* enzymatic hydrolysis and fermentation among wheat straw (**WS**), soybean hulls (**SBH**), and corn DDGS among 16 sources of each ingredient.

MATERIALS AND METHODS

Sample collection

A total of 48 samples were collected between May and July 2013; including 16 sources of WS, 16 sources of SBH, and 16 sources of corn DDGS (Table 3.1). The WS samples were obtained from University of Minnesota Beef Barn (St. Paul, MN), Nutrena (Minneapolis, MN), Dairyland Laboratories (Arcadia, WI), and University of Minnesota West Central Research and Outreach Center (Morris, MN). The SBH samples were

obtained from Nutrena (Minneapolis, MN), Archer Daniels Midland (Mankato, MN; Mexico, MO; Quincy, IL; Des Moines, IA; Fosteria, OH), AGP Ag Processing Inc. (Dawson, MN), Bunge (Council Bluffs, IA and Decatur, IN), and Consolidated Grain & Barge Soybean Processing (Mount Vernon, IN). Corn DDGS samples were obtained from a previous study (Kerr et al., 2013) and Highwater Ethanol (Lamberton, MN).

Enzymatic hydrolysis

All samples were ground to pass through a 1 mm mesh screen in a Wiley No. 4 Laboratory Mill (Arthur H. Thomas, Philadelphia, PA). The first 2-steps of the pepsin and pancreatin hydrolysis followed the procedures developed by Boisen and Fernandez (1997), and subsequent steps followed modifications by Jha et al. (2011a,b). Briefly, 2 g of each sample (6 runs × 1 replicate per run) were weighed into each 500 mL conical flask and incubated at 39^{o} C in a water bath. One hundred mL of phosphate buffer solution (0.1 *M*, KH_2PO_4: Na_2HPO_4 = 7:1, pH = 6.0) and 40 mL 0.2 *M* HCl solution (pH = 2.0) were added to each replicate. The pH was adjusted to 2.0 by 1 *M* HCl or 1 *M* NaOH. Two mL of 5 mg/mL chloramphenicol (C0378; Sigma-Aldrich Corp., St. Louis, MO) solution (dissolved in ethanol) were added to prevent bacterial growth during hydrolysis. Then each replicate was treated with 4 mL of 100 mg/mL fresh porcine pepsin (P7000, 421 units/mg solids; Sigma-Aldrich Corp.) solution (dissolved in 0.2 *M* HCl) at 39^{o} C and incubated in a water bath for 2 h, while all the flasks were manually shaken gently for 5 sec every 15 min. Afterward, 40 mL of 0.2 *M* phosphate buffer (KH_2PO_4: Na_2HPO_4 = 7:1, pH = 6.8) and 20 mL of 0.6 *M* NaOH were added to each flask. The pH was adjusted to 6.8 with 1 *M* HCl or 1 *M* NaOH and 4 mL of 100 mg/mL

fresh porcine pancreatin (P1750, 4 × USP specifications; Sigma-Aldrich Corp.) solution (dissolved in 0.2 *M* phosphate buffer) was added. The hydrolysis continued for 4 h under the same conditions as pepsin hydrolysis.

After enzymatic hydrolysis, residues were collected by filtration (40 µm filter paper; VWR International, Radnor, PA), washed with distilled water, ethanol (2 × 20 mL, 95%) and acetone (2 × 20 mL, 99.5%), dried for 72 h at 55^{o} C, and weighed for determination of IVDMD. To obtain sufficient residues for the subsequent *in vitro* fermentation, 4 to 8 replicates of the enzymatic hydrolysis procedure were conducted depending on the amount of residue remaining (Table 3.2).

In vitro fermentation

The rate and amount of *in vitro* fermentation of the hydrolyzed residues was assessed by a cumulative gas production technique (Bindelle et al., 2007; Bindelle et al., 2009; Jha et al., 2015). Briefly, the hydrolyzed residues from enzymatic hydrolysis of the same sample were pooled for *in vitro* fermentation. Blank inocula without substrates were used as controls. There were a total of 3 runs with 2 replicates per run of blanks, and hydrolyzed residues of WS, SBH, and corn DDGS (Table 3.2). About 0.2 g of each mixed hydrolyzed residue was weighed, all blanks and hydrolyzed residues of WS, SBH, and corn DDGS were incubated at 39^{o} C in a 125 mL rubber stoppered serum bottle with 30 mL buffer solution, and included macro and micro-minerals (Menke and Steingass, 1988), and fecal inoculum. Fecal inoculum was obtained from 5 growing pigs (19 to 21 wk age; 68.5 to 83.4 kg in BW; Hampshire × Yorkshire) from University of Minnesota St. Paul Campus Swine Teaching and Research Facility. Pigs had the same genetic

background and were fed a standard commercial corn-soybean meal diet without antibiotics (Maverick Nutrition Inc., Austin, MN). Fecal samples were collected randomly from 3 out of the 5 pigs immediately after pigs defecated. Feces were immediately pooled and placed in Ziploc bags after collection, all air was removed, and bags were sealed and kept at 39^{o}C and delivered to the laboratory within 30 min. The inoculum was prepared by diluting blended feces in the inoculation solution that was composed of distilled water (474 mL/L), trace mineral solution (0.12 mL/L, composed of $CaCl_2$ 132 g/L, $MnCl_3 \cdot 4H_2O$ 100 g/L, $CoCl_2 \bullet 6H_2O$ 10 g/L, and $FeCl_3 \bullet 6H_2O$ 80 g/L), *in vitro* buffer solution (237 mL/L, composed of NH_4HCO_3 4.0 g/L and $NaHCO_3$ 35 g/L), macro-mineral solution (237 mL/L; composed of Na_2HPO_4 5.7 g/L, KH_2PO_4 6.2 g/L, $MgSO4 \cdot 7H_2O$ 0.583 g/L, and NaCl 2.22 g/L) and resazurin (Blue dye, 0.1% w/v solution; 1.22 mL/L) and filtered through folded cheesecloth. The final inoculum concentration was 0.05 g feces per mL of buffer. Thirty milliliter of inoculum was transferred into bottles containing the hydrolyzed residues, and the bottles were sealed with rubber stoppers and placed in a water bath of 39^{o}C for incubation. Through the whole process, oxygen contact was avoided in inoculum preparation until the incubation step by adding reducing solution (distilled water 47.5 mL/L, 1 *M* NaOH 2 mL/L, Na_2S 335 mg/L) into the buffer solution and CO_2 (Jha et al., 2011a,b; Jha et al., 2015).

The gas produced during fermentation was measured at 2, 5, 8, 12, 16, 20, 24, 30, 36, 48 and 72 h through an inverted 25 mL burette with its stopcock end attached to vacuum and its open end submerged into a 39^{o} C water bath. Before assembling the burette apparatus, the headspace volume of the burette was determined. To measure gas

volume at each time point, the inverted burette was filled with water to remove the air, then the serum bottle was quickly transferred from the incubation water bath to the water bath with the measuring burette, and a 20 gauge needle was inserted through the rubber stopper. At each gas measurement time point, the operator opened the valve to release all of the gas into burette, and immediately recorded the volume displaced by the gas produced in the bottle using burette calibration marks. Once the measurement was recorded, the bottles were transferred back into the incubating water bath immediately. After *in vitro* fermentation, the residues were collected by filtration, washed, dried and weighed following the same procedures described for the hydrolyzed residues.

Physicochemical analysis

All feed ingredient samples were analyzed at the University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO). Chemical analyses (Table 3.1) were performed according to standard AOAC (2006) procedures using the following methods: DM (Method 930.15), ADF (Method 973.18), NDF (Method 2002.04), and TDF (Method 991.43).

Calculations

Hemicellulose. Hemicellulose, % = NDF, % - ADF, % [1]

Total feces needed to prepare the inoculum per run. Feces, g = 30 mL × No. of samples × No. of replicates per run × 0.05 g/mL [2]

Gas volume released at each time point. $V, mL = V_h + (25 – V_r)$, [3]

where, V_h is the volume of burette headspace, V_r is the reading volume record, $V_h \leq V \leq V_h + 25$; $V = V_r$, [4]

where, $0 < V < V_h$, V_r is measured the same as headspace volume;

$$V = V_{r1} + V_{r2} + \ldots + V_{rn}, \quad [5]$$

where, $V > V_h + 25$, shut down the valve before gas went beyond the open end of the burette, recorded V_{r1}, and then repeated the procedure the second time and recorded V_{r2}, repeated the procedure n^{th} time until finish recording all the produced gas.

In vitro dry matter digestibility from simulated gastric and small intestinal hydrolysis (**$IVDMD_h$**). The $IVDMD_h$ was calculated as follows: $IVDMD_h$, % = [(dry weight of the sample before hydrolysis – dry weight of residues)/dry weight of the sample before hydrolysis] × 100 [6]

In vitro dry matter digestibility from simulated large intestine fermentation (**$IVDMD_f$**). The $IVDMD_f$ was calculated as follows: $IVDMD_f$, % = [(dry weight of hydrolyzed residues – dry weight of the residues after fermentation)/ dry weight of hydrolyzed residues] × 100 [7]

In vitro dry matter digestibility from simulated total tract digestion (*$IVDMD_t$*). The $IVDMD_t$ was calculated as follows: $IVDMD_t$, % = $[1 – (1 – IVDMD_h/100) \times (1 – IVDMD_f/100)] \times 100$ [8]

Kinetics of gas production. Gas accumulation curves recorded during the 72 h of fermentation were modified according to monophasic model from Groot et al., (1996):

$$G = A / (1 + (B^C/t^C)), \quad [9]$$

where G (mL/g DM substrate) denotes the amount of gas produced per g of DM incubated, A (mL/g DM) represents the asymptotic gas production, B (h) is the time after

incubation at which half of the asymptotic amount of gas has been formed, C is a constant determining the sharpness of the switching characteristic of the profile.

When calculating $IVDMD_h$, $IVDMD_t$ and accumulated gas production volume, all data were corrected by subtracting blank values from observed values.

Volatile fatty acids (VFA) and energy production. The amount of VFA and energy production was calculated from referenced VFA production (Jha et al., 2015). Since there is no reference VFA data from modified three-step procedure of WS and SBH, we were only able to calculate VFA and energy production for corn DDGS samples.

$$\text{VFA production, mmol} = (A/200\ \text{mL}) \times VFA_r, \text{mmol}, \quad [10]$$

where, 200 mL represents the maximum gas production volume and the VFAr is referenced VFA production (Jha et al., 2015), acetate is 3.92 mmol/g DM of fermented substrate, propionate is 1.61 mmol/g DM of fermented substrate, and butyrate is 0.57 mmol/g DM of fermented substrate.

$$\text{Total energy production from VFA, kcal} = (\text{acetate, mmol}) \times 209.6\ \text{cal/mmol} + (\text{propionate, mmol}) \times 366.4\ \text{cal/mmol} + (\text{butyrate, mmol}) \times 522.2\ \text{cal/mmol} \quad [11]$$

where, 209.6 cal/mmol, 366.4 cal/mmol, and 522.2 cal/mmol are the amount of energy produced from acetate, propionate, and butyrate, respectively (Christensen et al., 1999).

$$\text{Corn DDGS, cal/g DM of corn DDGS} = \text{total energy production from VFA, kcal} \times (1-IVDMD_h) \quad [12]$$

Statistical analyses

The kinetics of gas production parameters were modeled using PROC NLIN procedure of SAS 9.3 (SAS Inst. Inc., Cary, NC). The IVDMD and fitted gas production kinetic parameters were analyzed using the PROC MIXED procedure of SAS 9.3.

The comparisons among the 3 high fiber ingredients (WS, SBH, and corn DDGS), as well as the comparisons within 16 sources nested under each ingredient were analyzed using the following linear additive model:

$$Y_{ijk} = \mu + \tau_i + \alpha_{j(i)} + \beta_k + \varepsilon_{ijk}, \quad [13]$$

where, Y is the parameter to be tested ($IVDMD_h$, $IVDMD_f$, and gas production kinetic parameters A, B, and C), μ is the overall population mean, τ_i is the effect of the i^{th} (i = 1, 2, 3) ingredient, $\alpha_{j(i)}$ is the effect of the j^{th} (j = 1, 2, 3,……,16) source nested under each ingredient, β_k is the effect of run (k = 6 for $IVDMD_h$, k = 3 for $IVDMD_f$ and $IVDMD_t$, A, B, and C), and ε_{ijk} = experiment error. The ingredient (n = 3), and source (n = 16) nested under each ingredient were fixed factors and the run was a random factor. The Least Square Means within 16 sources nested under each ingredient were analyzed by slice effect. Differences were considered significant when $P \leq 0.05$ and a trend when $0.05 < P < 0.1$.

Correlations between NDF, ADF, or TDF and IVDMD and asymptotic gas production were analyzed using PROC CORR procedure in SAS 9.3. Data were separated into 7 sets of variables: 16 sources of each high fiber ingredient (WS, SBH, and corn DDGS), 32 sources of 2 of the 3 high fiber ingredients (WS + SBH; SBH + corn DDGS; WS + corn DDGS), and all 48 sources of the 3 high fiber ingredients (WS + SBH + corn DDGS).

RESULTS

Variability in fiber composition among ingredients

The concentration of fiber, as measured by TDF, was greatest in WS, followed by SBH, and lastly corn DDGS (Table 3.1). Among sources of each ingredient the concentration of TDF varied less among sources of WS (CV = 4.9) and SBH (CV = 2.5) compared with corn DDGS (CV = 9.3). The concentration of fiber, as measured by NDF, was less than TDF for WS and SBH, but not for DDGS. The ratio between NDF and TDF was 0.84 and 0.83 for WS and SBH, and was 1.00 for DDGS. In SBH, hemicelluloses, accounted for a smaller fraction of NDF than in corn DDGS.

Variability in IVDMD among ingredients

The $IVDMD_h$ of corn DDGS was greater ($P < 0.01$) than SBH, which was greater ($P < 0.01$) than WS (Table 3.3). The $IVDMD_f$ of SBH was greater ($P < 0.01$) than corn DDGS, which was greater ($P < 0.01$) than WS. Therefore, the $IVDMD_t$ of corn DDGS was greater ($P < 0.01$) than SBH, which was also greater ($P < 0.01$) than WS. There were differences ($P < 0.01$) in $IVDMD_h$ within sources of WS, SBH, and corn DDGS. The CV among sources of WS and SBH was greater than for corn DDGS. There were no differences in $IVDMD_f$ within sources of WS or within sources corn DDGS, but there were differences among sources of SBH ($P = 0.01$).

Kinetics of gas production during in vitro fermentation

The asymptotic gas production (A) of SBH was greater ($P < 0.01$) than corn DDGS, which was greater ($P < 0.01$) than WS (Table 3.4). Likewise, time to half

asymptote (B) was reached faster ($P < 0.01$) among samples of SBH than corn DDGS and WS, but there were no differences between WS and corn DDGS.

Correlations of fiber composition, IVDMD, and asymptotic gas production

The greater concentration of fiber in each ingredient (as measured by TDF, NDF, or ADF) is, the smaller the observed $IVDMD_h$ is (Table 3.5). However, the concentration of ADF and NDF, but not TDF, was negatively correlated with $IVDMD_h$ in corn DDGS. The concentration of TDF, NDF, or ADF was not correlated with disappearance of DM during *in vitro* fermentation ($IVDMD_f$) or asymptotic gas production. But the correlation of $IVDMD_f$ and asymptotic gas production with concentration of dietary fiber depends on the specific type of ingredient. For example, $IVDMD_f$ was not correlated with concentration of TDF, ADF, or NDF of single ingredient WS, SBH, or corn DDGS. However, $IVDMD_f$ negatively correlated with concentration of TDF, ADF, and NDF of some ingredients like WS + SBH and WS + corn DDGS; $IVDMD_f$ was positively correlated with concentration of TDF, ADF, and NDF of some other ingredients like SBH + corn DDGS. The asymptotic gas production Also, $IVDMD_f$ is positively correlated with A of WS + SBH, SBH + corn DDGS, WS + corn DDGS, and WS + SBH + corn DDGS.

Variability of VFA and energy production of corn DDGS

The calculated VFA production ranged from 5.6 to 7.5 mmol/g DM of fermented corn DDGS with a CV of 9.5%. The energy calculated from the VFA produced was 17.2 to 27.3% of DE and 18.3 to 28.9% of ME in corn DDGS samples with a CV of 14.1%.

DISCUSSION

The working hypothesis of this experiment was that *in vitro* hydrolysis and fermentation of DM of ingredients with high fiber content are different not only among ingredients, but also within sources of the same ingredient. The data from this experiment confirmed the hypothesis that *in vitro* digestibility of DM at the hydrolysis step differs among feed ingredients, which is partially due to differences in the concentration of TDF. The lowest $IVDMD_h$ was observed in WS, followed by SBH, and the greatest digestibility was observed for DDGS. This $IVDMD_h$ was inversely proportional and negatively correlated with the concentration of TDF (r = - 0.99), NDF (r = - 0.99), and ADF (r = - 0.99) in these samples. These high correlations are a result of minimal hydrolysis of fiber during incubation of WS, SBH, and DDGS with pepsin (pH 2) and pancreatin (pH 6.8). However, there was no association between the $IVDMD_f$ and fiber content as measured by TDF, NDF, or ADF for these 3 ingredients (Table 3.5). Contrary to $IVDMD_h$, $IVDMD_f$ is not dependent on the concentration of fiber in ingredients. Ingredients such as WS and SBH have high content of fiber (i.e. TDF, NDF, and ADF), but fermentability of this fiber is much greater in SBH than WS. This observation suggests that factors other than concentration of fiber affect the fermentability of fiber. These data also suggest that inputs of TDF, NDF, or ADF without an estimate of ATTD or hindgut fermentation, are insufficient for prediction of ME or NE among high fiber feed ingredients.

The value of fitted kinetic parameters or *in vitro* DM digestibility of high fiber ingredients appears to depend on the type of feed ingredient being evaluated. Among sources of WS, there was a smaller CV of $IVDMD_f$ than observed among sources of SBH

or DDGS. Therefore, improvement in precision of ME or NE from estimating ATTD of TDF appears of less value among sources of WS than for sources of SBH or DDGS.

There are several factors that may affect *in vitro* digestibility of DM estimates.

Fiber solubility

There are different dietary fiber analysis methods. The NDF (sum of cellulose, hemicellulose, and lignin) and ADF (sum of cellulose and hemicellulose) are measured by detergent fiber procedure without recovering soluble fiber fractions like pectins, gums, and glucans (Grieshop et al., 2001). However, soluble fibers fractions as well as insoluble fiber fractions can be measured in TDF procedure (Method 991.43; AOAC, 2006). According to studies reported in the literature, fiber composition in WS, SBH, and DDGS is mainly insoluble. However, WS, SBH, and corn DDGS vary in their relative concentrations of insoluble fiber: where WS (98-99%; Panthapulakkal et al., 2006; Alemdar and Sain, 2008) and corn DDGS (95-100%; Urriola et al., 2010) contain more insoluble fiber than SBH (83-94%; Cole et al., 1999). It has been known that the more insoluble fiber is present in an ingredient source, the less fermentable the fiber is in the ingredient. However, it is not clear if differences in the amount of soluble fiber contribute to the differences in fermentability because these ingredients are comprised predominantly of insoluble fiber sources. The observation that $IVDMD_h$ differs among sources of the same ingredient suggest that factors other than just the concentration of TDF modify $IVDMD_h$.

Physical structure and lignin content of fiber

The extent of fiber fermentation is quite variable and depends on the accessibility of fiber to the microbial population in the hindgut (Oakenful, 2001). Lignification of fiber is an important structural factor that affects fiber accessibility (Jung et al., 1997). The concentration of lignin has a negative effect on nutrient digestibility because lignin is not digested in the GIT and its structure traps other nutrients preventing access to and contact by endogenous digestive enzymes (Jung et al., 1997). Therefore, differences in lignin concentration may partially explain the differences in fiber fermentability, where WS (15.9% lignin; Tamaki and Mazza, 2011) > SBH (2.6% lignin; Matkovic et al., 2010) and corn DDGS (2.6% lignin; NRC, 2012).

VFA and energy produced from dietary fiber fermentation

The VFA produced during fermentation can be absorbed by the epithelial cells and metabolized, supplying energy to the host (Bergman, 1990). The average supply of energy from VFA used for maintenance has been reported to be 15-24% (Dierick et al., 1989; Yen et al., 1991). In the current study, we used experimental conditions comparable to those reported by Jha et al. (2015) for corn DDGS fermentation. Specifically, NDF content in corn DDGS used in our experiment was 34% compared with 32% reported by Jha et al. (2015), which led to comparable $IVDMD_h$ (our study: 55.7% versus reference: 59.6%), $IVDMD_f$ (our study: 52.7% versus reference: 53.4%), and asymptotic gas production (our study: 208 versus reference: 200). Therefore, we referenced the VFA profile (Jha et al., 2015) of corn DDGS based on the asymptotic gas production, to further calculate the amount of energy produced from per gram of DM corn DDGS, and the ratio of energy produced from VFA to corn DDGS DE and ME fed

to pigs (Table 3.6). The range of the ratio is supported by other studies (Anguita et al., 2006; Iyayi and Adeola, 2015). *In vitro* studies have shown that diets containing 24% nonstarch polysaccharides (NSP) from barley and sugar beet pulp contributed 17.6% of the energy available for DE (Anguita et al., 2006), and diets containing 15% NDF from wheat bran, contributed 24.7% of available energy from fermentation to DE (Iyayi and Adeola, 2015). Therefore, energy produced from fiber ingredient fermentation in the large intestine can contribute a significant amount of DE for utilization by pigs. However, the precision of using this modified three-step procedure to estimate energy contributions to ME need to be evaluated in future studies.

Accuracy of current procedure to estimate digestible dietary fiber

To test the accuracy of the current procedure, we conducted a regression analysis of ATTD of NDF and ADF using 12 DDGS samples from previous data (Kerr et al., 2013) relative to $IVDMD_f$ (Figure 3.1), as well as a regression of ATTD of DM (Kerr et al., 2013) with $IVDMD_t$ (Figure 3.2). Both ATTD of NDF and ADF had significant regression with *in vitro* DM digestibility from the large intestine, but the R^2 were not very high. This confirmed that the use of the current three-step procedure to predict ATTD of dietary fiber may be feasible with some refinements. On the other hand, ATTD of DM was not accurately predicted by the *in vitro* total tract DM digestibility in the current study.

Necessity of dynamic prediction of digestible dietary fiber

When developing energy prediction equations, the chemical composition (e.g. NDF, ADF, TDF) only partially represent the portion of the nutrients digested and

absorbed in the intestine. A more precise estimate of the portion of nutrients digested and absorbed in the swine intestine is estimated with digestible nutrients (NRC, 2012). However, *in vivo* animal experiments are time- and cost-consuming, so using modified three-step procedure is a promising method to replace *in vivo* nutrient digestibility studies as inputs to energy prediction equations for high fiber ingredients.

CONCLUSION

The modified three-step procedure was useful for detecting the variability of DM digestibility among and between WS, SBH, and corn DDGS sources.

Table 3.1. Analyzed composition of wheat straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS), DM basis

Item		**WS[1]**	**SBH[1]**	**Corn DDGS[1]**
TDF[2]	Mean, %	90.8	78.9	34.4
	Range, %	81.8-99.8	74.6-82.1	30.8-44.1
	SD, %	4.4	1.9	3.2
	CV, %	4.9	2.5	9.3
NDF	Mean, %	76.5	65.5	34.3
	Range, %	69.0-83.4	60.9-67.7	28.8-44.0
	SD, %	3.7	1.8	4.0
	CV, %	4.8	2.8	11.7
NDF/TDF	Mean, %	84.1	83.0	100.6
	Range, %	81.7-86.6	80.6-85.6	69.2-127.3
	SD, %	1.4	1.2	13.7
	CV, %	1.7	1.5	13.6
ADF	Mean, %	54.9	49.6	11.2
	Range, %	51.5-59.7	47.8-51.9	8.6-15.0
	SD, %	1.8	1.2	2.0
	CV, %	3.3	2.5	17.6
Hemicellulose[3]	Mean, %	21.6	15.9	23.1
	Range, %	15.1-30.2	12.3-19.0	15.5-30.0
	SD, %	3.7	1.4	3.3
	CV, %	17.2	8.9	14.2

[1]Data represent 16 sources each of WS, SBH, and corn DDGS. There is 1 lab run per sample.

[2]TDF = Total dietary fiber.

[3]Calculated as NDF - ADF.

Table 3.2. The number of runs and replicates of the modified three-step procedure for wheat straw (WS), soybean hulls (SBH), corn distillers dried grains with solubles (DDGS), and blanks

Item	2-step enzymatic hydrolysis		Fermentation	
	Run[1]	Replicates per run	Run	Replicates per run
WS	4-6	1	3	2
SBH	5-6	1	3	2
DDGS	6-8	1	3	2
Blank			3	2

[1]The number of runs was determined by the amount of residues of each sample, with the goal of obtaining a sufficient amount of residue for fermentation.

Table 3.3. Variability in IVDMD of wheat straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS)[1]

Item		WS	SBH	Corn DDGS	*P*-value[2]
$IVDMD^{3}_{h}$, %	Mean, %	14.5[a]	19.7[b]	55.7[c]	< 0.01
	Range, %	11.2-18.3	16.7-23.0	45.3-63.2	
	SD,%	1.5	4.1	3.5	
	CV, %	10.5	20.9	6.3	
	P-value[4]	< 0.01	< 0.01	< 0.01	
$IVDMD^{5}_{f}$, %	Mean, %	41.8[a]	68.5[c]	52.7[b]	< 0.01
	Range, %	36.8-48.0	49.0-83.2	41.4-64.2	
	SD,%	3.5	8.2	5.9	
	CV, %	8.4	11.9	11.3	
	P-value	0.98	0.01	0.41	
$IVDMD^{6}_{t}$, %	Mean, %	50.2[a]	74.8[b]	79.2[c]	< 0.01
	Range, %	44.6-56.3	59.7-86.7	76.0-83.5	
	SD,%	3.3	6.4	2.0	
	CV, %	6.5	8.5	2.5	
	P-value	0.86	< 0.01	1.00	

[1]Data represent 16 sources each of WS, SBH, and corn DDGS.

[2]Refers to the comparison of the least square mean value among WS, SBH, and corn DDGS.

[3]$IVDMD_h$ = *in vitro* DM digestibility from simulated gastric and small intestinal hydrolysis.

[4]Refers to the comparison of the least square mean value within 16 sources of WS, SBH, or DDGS.

[5]$IVDMD_f$ = *in vitro* DM digestibility from simulated large intestine fermentation.

[6]$IVDMD_t$ = *in vitro* DM digestibility from simulated total tract digsestion.

[a,b,c]Means within rows with different superscripts are different ($P < 0.05$).

Table 3.4. Differences in fitted kinetic parameters on the gas accumulation recorded for wheat straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS) during *in vitro* fermentation

Item		**WS[1]**	**SBH[1]**	**Corn DDGS[1]**	***P*-value[2]**
A, mL/g substrate[3]	Mean	53.0[c]	293.0[a]	208.0[b]	< 0.01
	Range	32.0-80.0	262-324	183-246	
	SD	13.0	15.0	21.0	
	CV, %	24.3	5.2	9.8	
	P-value[4]	1.00	0.74	0.37	
B, h[5]	Mean	22.3[a]	14.5[b]	24.4[a]	< 0.01
	Range	12.1-40.2	13.3-15.9	17.9-33.8	
	SD	7.8	0.7	4.5	
	CV, %	35.1	4.5	18.5	
	P-value	< 0.01	1.00	0.40	
C,	Mean	1.87[c]	2.54[a]	1.30[b]	< 0.01
dimentionless[6]	Range	1.14-3.40	2.36-2.74	1.10-1.58	
	SD	0.62	0.1	0.17	
	CV, %	33.3	4.1	12.8	
	P-value	0.01	1.00	1.00	

[1]Data represent 16 sources each of WS, SBH, and corn DDGS.

[2]Refers to the comparison among WS, SBH, and corn DDGS.

[3]A = the amount of asymptotic gas production of substrate DM expressed as mL/g.

[4]Refers to the comparison within 16 sources of WS, SBH, or corn DDGS.

[5]B = the time in hours after incubation at which half of the asymptotic amount of gas has been produced.

[6]C = a constant that determines the sharpness of the switching characteristic of the gas profile.

[a, b, c]Means wihtin rows with different superscripts are different ($P \leq 0.05$).

Table 3.5. Correlations among fiber composition, *in vitro* dry matter digestibility and asymptotic gas production in 3 high fiber ingredients: wheat straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS)[1]

Item	Ingredients	TDF[2]	NDF	ADF	IVDMD[3]$_f$, %
IVDMD[4]$_h$, %	WS	-0.79*	-0.82*	-0.42	
	SBH	-0.65*	-0.74*	-0.78*	
	Corn DDGS	-0.22	-0.72*	-0.73*	
	WS + SBH	-0.92*	-0.93*	-0.86*	
	SBH + corn DDGS	-0.98*	-0.99*	-0.99*	
	WS + corn DDGS	-0.99*	-0.99*	-0.99*	
	WS + SBH + corn DDGS	-0.98*	-0.98*	-0.99*	
IVDMD$_f$, %	WS	-0.08	-0.04	0.20	
	SBH	0.23	0.11	0.02	
	Corn DDGS	-0.14	0.41	0.48†	
	WS + SBH	-0.77*	-0.79*	-0.77*	
	SBH + corn DDGS	0.74*	0.76*	0.75*	
	WS + corn DDGS	-0.76*	-0.71*	-0.72*	
	WS + SBH + corn DDGS	-0.07	-0.11	0.00	
A[5]	WS	-0.15	-0.14	0.15	0.27
	SBH	0.26	0.25	0.44†	-0.01
	Corn DDGS	-0.14	0.41	0.48†	-0.64
	WS + SBH	-0.86*	-0.88*	-0.84*	0.90*
	SBH + corn DDGS	0.91*	0.89*	0.92*	0.71*
	WS + corn DDGS	-0.97*	-0.97*	-0.97*	0.76*
	WS + SBH + corn DDGS	-0.34*	-0.40*	-0.27†	0.84*

[1]Pearson correlation coefficients (r).

[2]TDF = total dietary fiber.

[3]IVDMD$_f$ = *in vitro* DM digestibility from simulated large intestine fermentation.

[4]IVDMD$_h$= *in vitro DM* digestibility from simulated gastric and small intestinal hydrolysis.

[5]A = asymptotic gas production expressed as mL/g of DM of substrate.

*Significant correlation ($P \leq 0.05$)

†Correlation trend ($0.05 < P < 0.10$).

Table 3.6. Variability of VFA and energy production during *in vitro* fermentation of corn distillers dried grains with solubles (DDGS) using pig fecal inocula

Item	Mean	Range	SD	CV, %
VFA production, mmol/g DM of fermented substrate[1]	6.4	5.6-7.5	0.6	9.5
Energy production, cal/g DM of fermented substrate[2]	1,781.4	1,561.7-2,106.3	169.6	9.5
Energy production from corn DDGS, cal/g DM	789.0	627.6-962.1	106.3	13.5
Energy from VFA: DE[3] of corn DDGS, %	21.7	17.2-27.3	3.1	14.1
Energy from VFA: ME[4] of corn DDGS, %	23.1	18.3-28.9	3.3	14.1

[1]Fermented substrate was the hydrolyzed residue from pepsin and pancreatin hydrolysis (Jha et al., 2015).

[2]Fermented substrate was the hydrolyzed residue from pepsin and pancreatin hydrolysis (Christensen et al., 1999).

[3,4]Two samples were referenced from previous DE and ME values reported by Kerr et al. (2013), 4 samples were referenced from NRC (2012) for corn DDGS containing > 6% and < 9% ether extract (ME = 3,396 kcal/kg, DE = 3,582 kcal/kg) and corn DDGS containing > 10% ether extract (ME = 3,434 kcal/kg, DE = 3,620 kcal/kg).

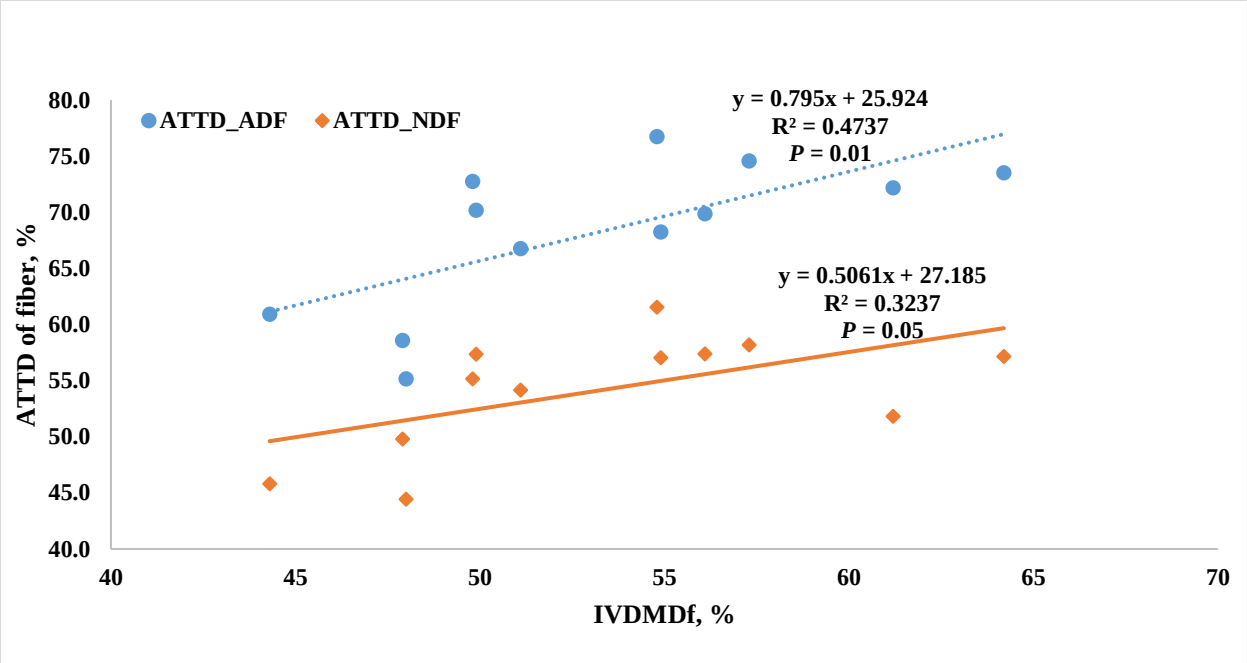

Figure 3.1. Regression of ATTD of NDF and ADF with $IVDMD_f$, where ATTD = apparent total tract digestibility and $IVDMD_f$ = *in vitro* large intestine fermentation disappearance.

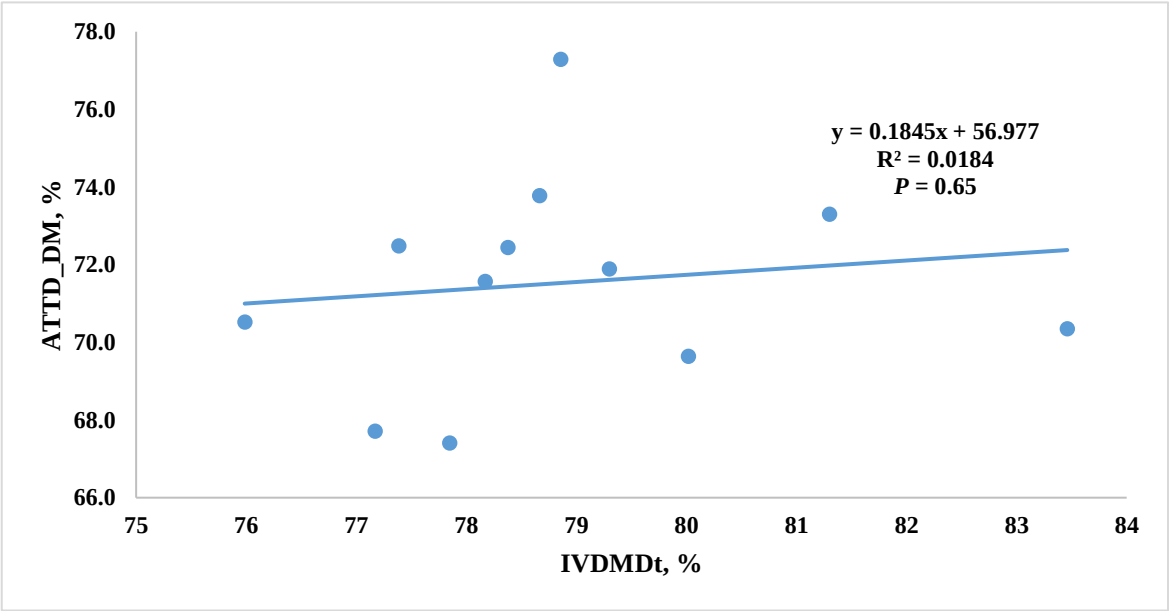

Figure 3.2. Regression of ATTD of DM with IVDMDt, where ATTD = apparent total tract digestibility and IVDMDt = *in vitro* DM digestibility from total tract.

Chapter 4. Use of *in vitro* DM digestibility and gas production to predict apparent total tract digestibility of total dietary fiber for growing pigs[1]

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ABSTRACT: *In vitro* dry matter digestibility (IVDMD) and gas production methods have been developed and evaluated to estimate *in vivo* nutrient digestibility of some feed ingredients, but further validation is needed. The aim of this study was to evaluate a three-step *in vitro* procedure and the resulting gas production to predict *in vivo* total dietary fiber (TDF) digestibility of what straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS). A total of 34 barrows and 2 gilts (BW 84 ± 7 kg) were used in a change-over design to determine *in vivo* apparent total tract digestibility (ATTD) of 9 dietary treatments: 3 WS diets, 3 SBH diets, and 3 corn DDGS diets. The WS, SBH, or corn DDGS sources were the only ingredients containing fiber in each diet, and all diets were balanced to provide similar TDF concentrations (22.3%). There were 2 consecutive 13-d periods, each including a 10-d adaptation and a 3-d collection period, and 0.5% TiO_2 was added to each diet as indigestible marker. Pigs had ad libitum access to water and were fed an amount of feed equivalent to 2.5% of initial BW in each period. The *in vitro* experiment determined IVDMD and gas production of the 9 ingredients fed during the *in vivo* experiment. Gas production kinetics were fitted using a non-linear model, comparisons among and within ingredients or diets were analyzed using a mixed model, and the predictions were evaluated using correlations and regression equations. Results showed that differences ($P < 0.01$) in *in vivo* ATTD of TDF were observed among WS (26.7%), SBH (78.9%), and corn DDGS (43.0%), and also within sources of corn DDGS (36.0 vs. 49.8%). Differences ($P < 0.05$) in IVDMD from hydrolysis ($IVDMD_h$) were observed among WS (13.3%), SBH (18.9%), and corn DDGS (53.7%), and also within sources of WS (12.8 vs. 13.8%), SBH (17.0 vs.19.0 vs. 20.5%), and corn DDGS (52.0, 52.1 vs. 56.9%). Differences ($P < 0.05$) in IVDMD from

fermentation ($IVDMD_f$) were also observed among WS (23.3%), SBH (84.6%), and corn DDGS (69.6%), and within sources of WS (18.7 vs. 26.8%). Total tract IVDMD ($IVDMD_t$) of SBH (88.9%) and corn DDGS (86.1%) were greater ($P < 0.01$) than WS (33.5%), and differences ($P < 0.05$) of $IVDMD_f$ were observed among sources of WS (30.0 vs. 36.5%). Differences ($P < 0.01$) in asymptotic gas production (A, mL/g DM substrate) were observed among WS (121), SBH (412), and corn DDGS (317), and ATTD of TDF was highly correlated with $IVDMD_f$ and A. In conclusion, use of *in vitro* DM digestibility and gas production are promising predictors of ATTD of TDF.
Key words: apparent total tract digestibility, corn distillers dried grains with solubles *in vitro*, soybean hulls, wheat straw

INTRODUCTION

As the inclusion of high fiber ingredients in swine diets increases, managing the inherent variability in ME, NE, and nutrient content has become a big challenge for optimizing caloric and nutritional efficiency. Fiber fermentation in the large intestine of pigs can contribute a significant amount of energy to pigs, but it is quite variable and difficult to measure. Apparent total tract digestibility (**ATTD**) of total dietary fiber (**TDF**) of corn distillers dried grains with solubles (**DDGS**) varies from 29.3 to 57.0% (Urriola et al., 2010). The low fiber digestibility of corn DDGS is mainly due to the high (95 to 100%; Urriola et al., 2010) insoluble dietary fiber (**IDF**) content and less digestible fiber components such as hemicellulose and lignin (Kim et al., 2008). The concentration of dietary fiber does not greatly affect the digestibility of fiber, but affects the amount of digestible fiber. When developing ME prediction equations for growing pigs fed corn DDGS, the concentration of NDF or TDF (Kerr et al., 2013; Urriola et al., 2014) can be

used to improve the accuracy of prediction. However, improved prediction accuracy and precision may be possible by using digestible and fermentable NDF or TDF in these equations. Therefore, using estimates of digestible nutrients and fermentable carbohydrates may improve prediction accuracy of NE equations for high fiber ingredients (Kil et al., 2013). Also, *in vitro* organic matter (**OM**) digestibility has been used as a fast and accurate measurement to predict DE and NE of feed ingredients (Noblet and Jaguelin-Peyraud, 2007).

A modified three-step *in vitro* procedure has been used to measure IVDMD and total gas production of swine feed ingredients and includes pepsin hydrolysis, pancreatin hydrolysis, and fecal fermentation (Boisen and Fernández, 1997; Bindelle et al., 2007). The IVDMD method has been used to predict *in vivo* ATTD of GE and DM for swine among sources of barley (Regmi et al., 2008) and wheat (Regmi et al., 2009), but not for corn DDGS, soybean hulls (**SBH**), or wheat straw (**WS**). Also, no prediction of *in vivo* ATTD of TDF have been evaluated using the modified three-step procedure in corn DDGS. Therefore, the hypothesis of this study was that the modified three-step procedure can accurately predict *in vivo* ATTD of TDF by IVDMD and gas production kinetics of WS, SBH, and corn DDGS.

MATERIALS AND METHODS

All experimental procedures involving animals were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Sample collection and diet formulation

We collected 3 sources each of ingredients containing high amounts of IDF to provide a range in IVDMD and chemical composition: WS (98 to 99% IDF;

Panthapulakkal et al., 2006; Alemdar and Sain, 2008), SBH (83 to 94% IDF; Cole et al., 1999), and corn DDGS (95 to 100% IDF; Urriola et al., 2010). The WS samples were collected from the University of Minnesota St. Paul Campus Beef Barn (St. Paul, MN), UMore Park (Rosemount, MN), and Southern Research and Outreach Center (Waseca, MN). The SBH sources were obtained from Archer Daniels Midland at 3 different locations (Mexico, MO; Des Moines, IA; Valdosta, GA). The corn DDGS samples were from Heron Lake BioEnergy, LLC (Heron Lake, MN), Big River Resources (Dyersville, IA), and Commonwealth Agri Energy (Hopkinsville, KY). The chemical composition of each ingredient and source is shown in Table 4.1.

Nine experimental diets were mixed for an *in vivo* experiment (Table 4.2), and WS, SBH, or corn DDGS sources served as the only source of fiber in each diet. Diets were formulated to meet the nutrient requirement of 80 to 90 kg growing pigs (NRC, 2012) and contained about 12% TDF. Titanium dioxide (TiO_2) was added at 0.5% to all diets as an indigestible marker.

In vivo animal experiment

Thirty-four growing barrows and 2 gilts (BW 84 ± 7 kg, Large White × Danish Landrace) were housed individually in metabolism cages (198 cm × 84 cm × 71 cm) at the Southern Research and Outreach Center in Waseca, MN. The experiment used a change-over design, 36 pigs were allotted to 4 blocks based on initial BW to provide 9 pigs in each block, and all 4 blocks had balanced average BW. The study lasted 30 d, including 4 d for pigs to adapt to the metabolism cages when they were fed acommercial diet. In the subsequent 2 consecutive 13-d periods, experimental diets were fed for 13-day. Feces and urine were collected on the final 3-d. In each period, the 9 pigs within

each block were randomly fed 1 of 9 experimental diets. At the end of period 1, each diet was consumed by 4 pigs; at the end of period 2, each diet was consumed by 8 pigs, resulting in a total of 8 replicates per dietary treatment.

In the adaptation period, the commercial corn-soybean diets were gradually replaced by experimental diets. Depending on the type of experimental diet, it takes a few days for the pigs to adapt. Overall, it takes 1-2 days for pigs to get use to corn DDGS diets, it takes 1-4 days for pigs to get use to SBH diets, and it takes 3-10 days for pigs to get use to WS diets. Due to great volume and dry texture of WS diets, the WS diets were mixed with water before fed to pigs with an increasing amount until they totally adapt it. Pigs were provided an amount of their respective dietary treatments equivalent to 2.5% of their initial BW divided into 2 equal meals and fed twice daily (0800 and 1600 h). Water was available *ad libitum* from nipple drinkers. Pigs were weighed at the beginning and the end of each period, before the morning meal. Feces of each pig were collected twice daily at 0800 and 1600 h by using a fine wire mesh screen placed under the cage for 3 d during each collection period. About 200 g of feces per day were collected in sealed plastic bags and kept frozen (-20 ^{o}C) until further processing. At the conclusion of the 2 collection periods, fecal samples were weighed and oven dried at 60^{o}C for 4 d, ground through a 1-mm screen, and subsampled for further analysis.

In vitro dry matter digestion and gas production

All 9 sources of ingredient samples were ground to pass through a 1 mm screen in a Wiley No.4 Laboratory Mill (Arthur H. Thomas, Philadelphia, PA) for determining *in vitro* determination of DM digestibility and gas production. A modified three-step enzymatic and microbial fermentation procedure was used (Boisen and Fernández, 1997;

Bindelle et al., 2007). Briefly, 2 g of each sample was subjected to hydrolysis through the first 2-steps of this procedure with pepsin (100 mg/mL 0.2 M HCl; P7000, 421 units/mg solids; Sigma-Aldrich Corp.) for 2 h, and pancreatin (100 mg/mL 0.2 M phosphate buffer; P1750, 4 × USP specifications; Sigma-Aldrich Corp.) for 4 h. After enzymatic digestion, the residues were collected by filtration through a nylon bag (5 cm × 10 cm; pore size 50 µm; Ankom Technology, Macedon, NY), washed with distilled water, ethanol (2 × 20 mL, 95%), and acetone (2 × 20 mL, 99.5%), dried for 72 h at 55ºC, and weighed for determination of IVDMD. The residues of 5 to 8 digestion replicates were pooled for each sample, and 200 mg were used for the third step of this procedure involving microbial fermentation. Residues were incubated at 39ºC in a glass bottle with 30 mL buffer solution, including macro and microminerals (Menke and Steingass, 1988), and a swine fecal inoculum (Bindelle et al., 2007). The fecal inocula were prepared by pooling feces from 9 pigs representing each dietary treatment in the *in vivo* experiment. The inoculum was prepared by diluting blended feces with the macro and micro mineral buffer solution and filtered through folded cheesecloth. The final inoculum concentration was 0.05 g feces per mL of buffer. Each of the 30 mL inocula were transferred into bottles containing the digested residues, and the bottles were sealed with a rubber stopper and placed in 39 ºC water bath for incubation. Through the whole process (inoculum preparation until the incubation step), an anaerobic environment was maintained by adding reducing agents (1 M NaOH 0.2% and Na_2S, nonahydrate 0.335 g/L) into the buffer solution and CO_2 gas.

The amount of gas produced during fermentation was measured at 2, 5, 8, 12, 16, 20, 24, 30, 36, 48 and 72 h using an inverted 25 mL burette, with its stopcock end

attached to a vacuum line and its open end submerged in a 39°C water bath. Before assembling the burette apparatus, the headspace volume of the burette was determined. To measure the gas volume produced, the inverted burette was filled with water to remove the air, and then a 20 gauge needle was quickly inserted through stopper of the fermentation bottle and attached to the burette apparatus. The burette valve was opened to release the gas into the burette, and the volume displaced by the gas was immediately recorded. After *in vitro* fermentation, the residues were collected, filtered and washed as previously described for the residues, then dried for 72 h at 55°C and weighed for determination of IVDMD.

Physicochemical analysis

All samples were analyzed at a commercial lab (Omaha, NE). The analysis methods for ingredients, diets, and feces were as follows: DM (method 930.15, AOAC 2006), GE (ASTM D 5865-13), CP (method 992.15, AOAC 2006), ether extract with acid hydrolysis (EE; method 922.06, AOAC 2006), ADF (Ankom Technology), NDF (Ankom Technology), TDF (method 991.43), lignin (method 973.18, AOAC 2006), titanium (WDXRF), bulk density (USP <616> method I), and viscosity (Perten, AACC International Procedure RVA Method I).

Calculations

Hemicellulose. Hemicellulose, % = NDF, % - ADF, % [1]

Cellulose. Cellulose, % = ADF, % - Lignin, % [2]

Apparent total tract digestibility of TDF. ATTD, % = [(TDF in ingredient/TiO_2 in ingredient-TDF in feces/TiO_2 in feces)/(TDF in ingredient/TiO_2 in ingredient)] × 100 [3]

Total feces needed to prepare the inoculum per run. Feces, g = 30 mL × No. of samples

× No. of replicates per run × 0.05 g/mL [4]

Gas volume released from each time point. V, mL = V_h + (25 – V_r), [5]

where, V_h is the volume of the burette headspace, V_r is the reading volume record, $V_h \leq V \leq V_h + 25$;

$V = V_r$, [6]

where, $0 < V < V_h$, V_r is measured the same as headspace volume;

$V = V_{r1} + V_{r2} \ldots + V_{rn}$, [7]

where, $V > V_h + 25$, shut down the valve before gas went beyond the open end of the burette, recorded V_{r1}, and then repeated the procedure the 2nd time and recorded V_{r2}, repeated the procedure n^{th} time until finish recording all the produced gas. Based on our experience, V was rarely beyond the burette capacity.

Gastric and small intestinal hydrolysis disappearance. *In vitro* DM disappearance from simulated gastric and small intestinal hydrolysis (**$IVDMD_h$**) was calculated as follows: $IVDMD_h$, % = [(dry weight of the sample before digestion – dry weight of residues)/dry weight of the sample before digestion] × 100 [8]

Large intestine fermentation disappearance. *In vitro* DM disappearance from large intestine fermentation (**$IVDMD_f$**) was calculated as follows: $IVDMD_f$, % = [(dry weight of hydrolyzed residues – dry weight of the residues after fermentation)/ dry weight of hydrolyzed residues] × 100 [9]

Apparent total tract disappearance. *In vitro* DM disappearance from total gastrointestinal tract (**$IVDMD_t$**) was calculated as follows: $IVDMD_t$, % = [1 – (1 – $IVDMD_h$/100) × (1 – $IVDMD_f$/100)] × 100 [10]

Kinetics of gas production. Gas accumulation curves recorded during the 72 h of

fermentation were modified according to monophasic model from Groot et al., (1996):

$$G = A / (1+ (B^C/t^C)), \quad [11]$$

where G (ml/g DM substrate) denotes the amount of gas produced per gram of dry matter incubated, A (mL/g DM substrate) represents the asymptotic gas production, B (h) is the time after incubation at which half of the asymptotic amount of gas has been formed, C is a constant determining the sharpness of the switching characteristic of the profile.

Statistical analyses

The kinetics of gas production parameters were modeled using the PROC NLIN procedure of SAS 9.3 (SAS Inst. Inc., Cary, NC). The ATTD of TDF, IVDMD, and fitted gas production kinetic parameters were analyzed using the PROC MIXED procedure of SAS 9.3. The comparisons among the 3 IDF ingredients (WS, SBH, and corn DDGS), as well the comparisons within 3 sources nested under each ingredient were analyzed using the following linear additive model:

$$Y_{ijk} = \mu + \tau_i + \alpha_{j(i)} + \beta_k + \varepsilon_{ijk}, \quad [12]$$

where, Y is the parameter to be tested (ATTD of TDF, IVDMD or gas production kinetic parameters A, B and C), μ is the overall population mean, τ_i is the effect of the i^{th} (i = 1, 2, 3) ingredient or diet, $\alpha_{j(i)}$ is effect of the j^{th} (j = 1, 2, 3) sources nested under each ingredient, β_k is the effect of replicate (k = 8), ε_{ijk} = experiment error. The ingredient (n = 3) and source (n = 3) nested under each ingredient were fixed factors and the replicate was a random factor. The Least Square Means within 3 sources nested under each ingredient were analyzed using the slice effect. Differences were considered significant when $P \leq 0.05$ and a trend when $0.05 < P < 0.1$.

The PROC CORR was used to determine the associations between ATTD of TDF of experimental diets with IVDMD and A. Data were separated into 7 sets of variables: 3 sources of each ingredient (WS, SBH, and corn DDGS), 6 sources of 2 of the 3 ingredients (WS + SBH; SBH + corn DDGS; WS + corn DDGS), and all 9 sources of the 3 ingredients (WS + SBH + corn DDGS). PROC REG (stepwise) was used to determine the prediction equations of ATTD of TDF for WS, SBH, and corn DDGS from $IVDMD_f$ and A. The variables with $P \leq 0.15$ were retained in the model. Variance inflation and collinearity diagnostics were tested by COLLIN (Lamberson and Kaps, 2004) to avoid selection of highly correlated $IVDMD_f$ and A at the same time. The R^2, SE, C(p), and difference between predicted and measured ATTD of TDF were used to define the accuracy of the prediction equations.

RESULTS

Chemical composition of WS, SBH, and corn DDGS

In the current study, we selected WS, SBH, and corn DDGS as 3 ingredients with high concentration of TDF, but with different chemical compositions and fiber solubility (Table 4.1). The chemical composition was compared numerically instead of statistically. Overall, sources of corn DDGS had relatively greater GE, CP, EE, and bulk density than WS or SBH sources. Corn DDGS also had relatively greater particle size than sources of SBH. Sources of WS had comparable GE and EE to SBH, but less CP and bulk density.

The concentration of fiber fractions was also different among WS, SBH, and corn DDGS and within sources of the same ingredient. Sources of WS and SBH had relatively greater ADF, NDF, TDF, and cellulose than corn DDGS. Sources of SBH also had relatively lower NDF than sources of WS. The concentration of TDF within WS, SBH,

and corn DDGS sources varied from 77.2 to 82.6%, 75.9 to 80.2%, 37.5 to 38.2%, respectively. The concentration of NDF within WS, SBH, and corn DDGS sources varied from 78.2 to 80.0%, 63.2 to 68.4%, and 32.7 to 35.1%, respectively.

Apparent total tract digestibility of total dietary fiber

The ATTD of TDF in SBH was greater ($P < 0.01$) than in corn DDGS, which was also greater ($P < 0.01$) than in WS (Table 4.3). There were no differences in the ATTD of TDF among sources of WS or SBH. However, among sources of corn DDGS we observed a range in ATTD of TDF between 36.0% and 49.8%.

In vitro dry matter digestibility and gas production

The $IVDMD_h$, $IVDMD_f$, and $IVDMD_t$ were different among WS, SBH, and corn DDGS. Given the greater content of CP, EE, and starch in corn DDGS, we observed greater ($P < 0.01$) $IVDMD_h$ in corn DDGS than SBH and WS. Also, we observed greater ($P < 0.01$) $IVDMD_h$ in SBH than WS. The $IVDMD_f$ was greatest ($P < 0.01$) in SBH, intermediate in corn DDGS, and least ($P < 0.01$) in WS. The small $IVDMD_f$ combined with small $IVDMD_h$ of WS, resulted in the least ($P < 0.01$) $IVDMD_t$ of WS compared with SBH and corn DDGS; while there were no differences between corn DDGS and SBH. Differences of $IVDMD_h$ ($P = 0.05$), $IVDMD_f$ ($P < 0.05$), and $IVDMD_t$ ($P < 0.05$) within sources of WS were also observed. However, only differences of $IVDMD_h$ were observed within sources of SBH ($P < 0.01$) and corn DDGS ($P < 0.01$), but not for $IVDMD_f$ and $IVDMD_t$.

The differences in $IVDMD_f$ were well in agreement with the asymptote of the gas production curve (A, mL of gas produced), where SBH had greater ($P < 0.01$) A than corn DDGS, which in turn had greater ($P < 0.01$) A than WS. There were no differences

in asymptotic gas production within sources of WS, SBH, or corn DDGS.

Prediction of ATTD of TDF

In the current study, ATTD of TDF had a positive correlation ($P < 0.05$) with both $IVDMD_f$ and A for WS + SBH, SBH + corn DDGS, WS + corn DDGS, and WS + SBH + corn DDGS (Table 4.4). However, ATTD of TDF had no correlation with $IVDMD_f$ or A for WS or SBH or corn DDGS when the ingredients were analyzed separately.

The ATTD of TDF could be predicted by $IVDMD_f$ and A instead of using the more time consuming and expensive *in vivo* digestibility experiments. To test the accuracy of using $IVDMD_f$ and A as predictors, the prediction equations for ATTD of TDF were separate into 7 data sets among and within WS, SBH, and corn DDGS. Equations 1 to 7 were the prediction equations of ATTD for TDF of WS, SBH, and corn DDGS, WS + SBH, SBH + corn DDGS, WS + corn DDGS, and WS + SBH + corn DDGS based on their $IVDMD_f$ or A (Table 4.5). As indicated by the correlation, the ATTD of TDF could be predicted by $IVDMD_f$ and A for WS + SBH, SBH + corn DDGS, WS + corn DDGS, and WS + SBH + corn DDGS ($P < 0.05$), but not for WS, SBH, or corn DDGS. When separating each ingredient, the sample size was too small (n = 3) to develop accurate prediction equations. Consequently, the prediction equations had a limited number of parameters as predictors. The prediction equations for ATTD of TDF within sources of SBH were not possible because the difference in ATTD of TDF among sources was small. Among sources of WS and corn DDGS, the ATTD of TDF could be predicted from $IVDMD_f$ using equation 1 and 3. However, these predictions were not significant ($P > 0.05$) even though the R^2 were high, because the differences in measured value were small, and may have been due to small sample size.

When combining WS, SBH, and corn DDGS, ATTD of TDF could be predicted using equation (7) which contained the asymptotic gas production (A) with relatively high precision (R^2 = 0.82, SE = 10.68, C(p) = 3.13). This prediction equation had the lowest R^2 (0.82) but greatest differences (5.1 to 45.7%) between predicted and measured ATTD of TDF among the 7 equations.

DISCUSSION

The objective of this experiment was to test the hypothesis that *in vivo* ATTD of TDF can be predicted by an *in vitro* dry matter digestibility and gas production procedure. We selected an *in vitro* model developed by Boisen and Fernandez (1997) and modified by Bindelle et al. (2007) for its simplicity and relative frequency of use in previously published papers. Also, this *in vitro* model was used previously to predict the ATTD of NDF of rice bran, tofu residue, and water spinach (Dung and Udén, 2002) with relatively good success. However, none of these procedures were developed to predict ATTD of NDF in the large intestine of pigs fed diets with corn co-products, such as corn DDGS, SBH, and WS, nor are there data showing the accuracy of predicting ATTD of TDF among high fiber ingredients.

We selected ATTD of TDF because this measurement is a more reliable predictor of the carbohydrate disappearance in the large intestine of monogastric species than ATTD of NDF. Portions of soluble dietary fiber, such as pectins, gums, and glucans, are not recovered in the analysis of NDF (Mertens, 2003). Therefore, estimates of degradation of dietary fiber in the large intestine may not be accurate when using the NDF procedure. Also, the majority of agri-industrial by-products, such as corn DDGS, contain substantially greater proportion of insoluble dietary fiber compared with soluble fiber

(Stein and Shurson, 2009). As a result, it is necessary to evaluate the accuracy of the *in vitro* procedure for estimating ATTD of TDF among feed ingredients with relatively high concentrations of insoluble dietary fiber. Except solubility, viscosity and specific fiber composition are also affect fermentability of fiber (Nyman et al., 1986). Therefore, we selected 3 insoluble fiber sources with different fiber composition and viscosity: WS, SBH and corn DDGS.

In agreement with our hypothesis, the current *in vitro* model was useful in predicting the ATTD of TDF among all 3 feed ingredients with relatively high content of insoluble dietary fiber. First, the ATTD of TDF in WS and SBH were in agreement with expected values based on measurements of the ATTD of NDF and non-starch polysaccharides (Chabeauti et al., 1991) and based on the high degree of lignification of WS (Jung et al., 1997). Also, we expected a high (> 80%) ATTD of TDF in SBH based on previous observations (Urriola and Stein, 2012), along with previous observations of high ATTD of NDF (Kornegay, 1978; Kornegay, 1981). This high ATTD of SBH may be because of the relatively high solubility of SBH (6-17%; Cole et al., 1999) compared with WS (1-2%; Panthapulakkal et al., 2006; Alemdar and Sain, 2008) and corn DDGS (0-5%; Urriola, 2010). The main soluble fiber component in SBH is pectin (Snyder and Kwon, 1987). The values for disappearance of DM during *in vitro* fermentability using fecal inocula ($IVDMD_f$) for WS and SBH were in agreement with the measured ATTD of TDF. Intermediate (> 30 and < 60%) ATTD of TDF was expected for corn DDGS and observations from this experiment are in agreement with those previously reported by Urriola et al. (2010).

Using the *in vitro* disappearance of DM during the fermentation step ($IVDMD_f$), it

appears to be of similar benefit to using the measured asymptotic gas production value (A) to predict ATTD of TDF among all 3 high fiber feed ingredients. As shown in Table 4.5, most of the equations only use $IVDMD_f$ or A as parameters. Therefore, there appears to be minimal benefit of providing additional information from the rate of gas production (B) or the shape of the gas production curve (C) in predicting ATTD of TDF. Even though in equation (2)-1, B was used as a predictor for TDF digestibility of SBH, but the *P* value was not significant. Also, in equation (7)-1, the accuracy was improved by adding C to equation (7), but equation (7) also had significant *P* value. Therefore, B or C may be used in some of these prediction equations, but A or $IVDMD_f$ were the main predictor variables for TDF digestibility. These observations suggest that a portion of dietary fiber in all 3 feed ingredients remains indigestible regardless of the kinetics of degradation of dietary fiber, and belong to a recalcitrant portion of the dietary fiber (de Vries et al. 2013). The kinetics of degradation of dietary fiber along the gastrointestinal tract of pigs may have implications due to the interaction of dietary fiber with digestibility of other nutrients such as amino acids and lipids, but there are no data to confirm this hypothesis.

Despite good agreement between the *in vivo* and *in vitro* observations among the feed ingredients evaluated in this study, the accuracy of prediction was different among these 3 ingredients. Differences in the ATTD of TDF among sources of WS or SBH were relatively small, which was likely a result of the small range in TDF between the 3 sources of these 2 ingredients. There are 2 reasons that may explain this observation. Ingredients such as WS and SBH represent 2 examples of ingredients with low (< 30%) or high (> 80%) ATTD of TDF and it appears that there is little variability among sources

of these ingredients. In fact, the magnitude of ATTD of TDF and $IVDMD_f$ was similar and suggest that WS had low degradability under both evaluation conditions (*in vitro* and *in vivo*), while SBH had high degradability using both the *in vivo* and *in vitro* methods.

In contrast, the range in ATTD of TDF among sources of corn DDGS (36.0 to 49.8%) was greater than the range between WS and SBH. In fact, a larger range in ATTD of TDF (29.3 to 57.0%) has been reported (Urriola et al., 2010; Gutiérrez et al., 2014). Therefore, it appears that among sources of corn DDGS, which had intermediate ATTD of TDF, many factors such as source of corn, differences in processing methods used in various ethanol plants, or interactions among nutrients affect ATTD of TDF. Other conditions of retention time or incubation time appear to affect degradability of dietary fiber in corn DDGS, but not in WS or SBH. The difference between *in vitro* dry matter fermented (69.6%) and *in vivo* ATTD of TDF (43.0%) was greater in DDGS than WS or SBH. This observation is in agreement with previous observations on the ATTD of TDF among breeds of pigs, where longer retention time increases the ATTD of TDF in DDGS, but it did not affect ATTD of TDF of soluble dietary fiber, such as pectins (Udén and van Soest, 1982).

CONCLUSION

In conclusion, the results of this experiment indicate that it is promising to predict ATTD of TDF through $IVDMD_f$ or the asymptotic gas production value (A) for high fiber ingredients such as WS, SBH, and corn DDGS. Developing prediction equations for each ingredient was more accurate and practical than combining different high fiber ingredients together. Using static values for ATTD of TDF among sources of high fiber feed ingredients with low or high ATTD of TDF such as WS and SBH seems reasonable

based on the appearance that there is minimal variability in nutrient and fiber composition among sources of these ingredients. However, there appears to be large variability among sources of feed ingredients with intermediate ATTD of TDF, such as corn DDGS. For corn DDGS, using dynamic prediction values based on *in vitro* digestibility of DM or asymptotic gas production will improve nutritionists' ability to enhance caloric and nutritional efficiency when formulating swine diets using DDGS.

Table 4.1. Analyzed composition of wheat straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS), DM basis

Item	WS			SBH			Corn DDGS		
	1	2	3	4	5	6	7	8	9
GE, kcal/kg	4,050	4,160	4,032	4,070	4,065	3,998	4,879	4,842	4,776
CP, %	4.1	6.2	3.8	11.5	12.6	11.3	29.9	31.6	30.7
EE[1], %	2.2	2.5	2.8	2.4	2.8	2.8	9.8	9.0	8.2
ADF, %	53.0	53.0	52.7	51.0	48.2	50.2	13.8	17.4	14.5
NDF, %	79.1	80.0	78.2	68.4	63.2	66.4	32.7	35.1	33.8
TDF[2], %	77.2	82.6	80.0	80.2	75.9	75.9	38.2	37.5	37.8
NDF/TDF, %	102.5	96.9	97.8	85.3	83.3	87.5	85.6	93.6	89.4
Lignin, %	6.6	7.6	7.5	3.5	3.9	4.9	1.8	4.6	2.6
Hemicellulose[3], %	26.0	27.0	25.5	17.4	15.1	16.2	18.8	17.7	19.2
Cellulose[4], %	46.4	45.4	45.2	47.5	44.3	45.2	12.0	12.8	11.9
Bulk density, g/cm^3	0.18	0.19	0.16	0.39	0.42	0.41	0.48	0.50	0.54
Viscosity, centipoise	420	665	859	36	27	44	-30	4	-4
Particle size, μm	NA[5]	NA	NA	720	600	715	793	804	644

[1]Ether extract with acid hydrolysis.

[2]Total dietary fiber.

[3]Calculated as NDF-ADF.

[4]Calculated as ADF-lignin.

[5]Not applicable. The particle size of wheat straw could not be determined because of the long and rigid shape of ground particles.

Table 4.2. Ingredient composition and nutrient content of wheat straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS) diets, DM basis

Item	WS			SBH			Corn DDGS		
	1	2	3	4	5	6	7	8	9
Ingredient composition, %									
WS	23.00	23.00	23.00	0.00	0.00	0.00	0.00	0.00	0.00
SBH	0.00	0.00	0.00	30.00	30.00	30.00	0.00	0.00	0.00
Corn DDGS	0.00	0.00	0.00	0.00	0.00	0.00	55.00	55.00	55.00
Plasma spray-dried	4.73	4.73	4.73	4.73	4.73	4.73	4.73	4.73	4.73
Corn starch	61.10	61.10	61.10	56.66	56.66	56.66	34.73	34.73	34.73
Casein	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Fish meal, menhaden	6.74	6.74	6.74	3.77	3.77	3.77	0.00	0.00	0.00
Titanium dioxide	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Dicalcium phosphate (18.5%)	0.00	0.00	0.00	0.30	0.30	0.30	0.00	0.00	0.00
Limestone	0.32	0.32	0.32	0.44	0.44	0.44	1.44	1.44	1.44
Sodium chloride	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Grow-finishing VTM premix[1]	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Total	99.99	99.99	99.99	100.00	100.00	100.00	100.00	100.00	100.00
Analyzed nutrient composition, DM basis									
GE, kcal/kg	4,160	4,169	4,174	4,106	4,105	4,099	4,517	4,480	4,429
CP, %	13.00	13.60	12.40	13.20	13.70	13.10	22.20	24.00	23.00
EE[2], %	2.90	2.60	3.10	2.40	2.50	2.30	6.30	6.30	6.00
ADF, %	12.40	12.50	13.00	15.30	13.90	14.70	8.60	10.00	7.70
NDF, %	22.20	23.50	26.90	21.30	19.80	23.50	18.70	19.60	20.40
TDF[3], %	23.00	23.40	21.40	24.70	23.20	25.00	20.70	20.90	18.30
Lignin, %	2.20	2.30	2.20	0.80	0.90	1.50	2.00	2.80	2.10
Hemicellulose[4], %	9.70	11.00	13.90	6.00	5.90	8.80	10.00	9.60	12.70
Cellulose[4], %	10.30	10.20	10.80	14.50	13.00	13.20	7.00	7.20	5.60
Titanium, %	0.33	0.33	0.30	0.27	0.33	0.28	0.35	0.38	0.37
Bulk density, g/cm^3	0.37	0.42	0.40	0.64	0.61	0.60	0.67	0.68	0.67
Viscosity, centipoise	1,989	2,142	2,455	532	607	817	311	301	310

[1]The vitamin and trace mineral premix (ANS Swine G-F premix) provided the following (per kg of diet): Vitamin A 3,527,392 I.U., Vitamin D 3 661,386 I.U., Vitamin E 13,228 I.U., Vitamin K (MPB) 1,323 mg, Riboflavin 2,205 mg, Niacin 13,228 mg, Pantothenic Acid 8,818 mg, Vitamin B12 13 mg, Iodine (EDDI)

119 mg, Selenium (Selenite) 119 mg, SQM Organic Zinc 22,046 mg, SQM Organic Iron 13,228 mg, SQM Organic Manganese 454 mg, SQM Organic Copper 1,543 mg.

[2]Ether extract with acid hydrolysis.

[3]Total dietary fiber.

[4]Hemicellulose was calculated as NDF - ADF; cellulose was calculated as ADF - lignin.

Table 4.3. Apparent total tract digestibility (ATTD) of total dietary fiber (TDF), IVDMD, and kinetics of gas production of 3 insoluble dietary fiber ingredients: wheat straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS)[1]

Item	ATTD of TDF, %	IVDMD[2]$_h$, %	IVDMD[3]$_f$, %	IVDMD[4]$_t$, %	A[5]	B[6]	C[7]
WS	26.7^{c}	13.3^{c}	23.3^{c}	33.5^{b}	121^{c}	21.3^{ab}	1.98^{a}
SBH	78.9^{a}	18.9^{b}	84.6^{a}	88.9^{a}	412^{a}	20.0^{b}	2.15^{a}
Corn DDGS	43.0^{b}	53.7^{a}	69.6^{b}	86.1^{a}	317^{b}	22.6^{a}	1.45^{b}
SEM	2.24	0.24	1.83	1.46	11.7	1.03	0.12
P-value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.04	< 0.01
WS							
1	24.2	13.8^{A}	18.7^{B}	30.0^{B}	100	18.8^{B}	2.03
2	29.4	12.8^{B}	24.3^{AB}	33.9^{AB}	141	23.3^{A}	1.94
3	26.4	13.4^{AB}	26.8^{A}	36.5^{A}	122	21.9^{AB}	1.97
SEM	1.75	0.16	1.40	0.94	9.81	0.93	0.09
P-value	0.52	0.05	0.03	0.02	0.19	0.05	0.91
SBH							
4	79.0	17.0^{C}	86.8	92.4^{x}	407	20.2	2.31
5	75.0	20.5^{A}	84.1	88.1^{xy}	412	18.7	2.10
6	82.7	19.0^{B}	82.9	86.2^{y}	419	21.0	2.05
SEM	1.49	0.17	1.37	1.03	9.29	0.88	0.08
P-value	0.12	< 0.01	0.43	0.06	0.83	0.42	0.39
Corn DDGS							
7	43.1^{AB}	52.0^{B}	70.0	86.6	335	26.0^{A}	1.43
8	49.8^{B}	52.1^{B}	71.0	86.1	313	21.4^{B}	1.46
9	36.0^{A}	56.9^{A}	67.7	85.8	305	20.4^{B}	1.47
SEM	1.49	0.19	1.45	1.13	9.46	0.90	0.08
P-value	< 0.01	< 0.01	0.58	0.96	0.33	< 0.01	0.98

[1]Data were expressed as means.

[2]$IVDMD_h$: *In vitro* DM digestibility from simulated gastric and small intestinal hydrolysis.

[3]$IVDMD_f$: *In vitro* DM digestibility from simulated large intestine fermentation.

[4]IVDMDt: *In vitro* DM digestibility from simulated total tract digestion.

[5]A: mL/g DM substrate, represents the asymptotic gas production.

[6]B: h, the time after incubation at which half of the asymptotic amount of gas has been formed.

[7]C: A constant determines the sharpness of the switching characteristic of the profile.

[a,b,c] Expressed differences ($P \leq 0.05$) among different ingredients.

[A,B,C] Expressed differences ($P \leq 0.05$) and [x,y]expressed trends ($0.05 < P < 0.1$) of differences within different sources of the same ingredient.

Table 4.4. Correlations of apparent total tract digestibility (ATTD) of total dietary fiber (TDF) with IVDMD and asymptotic gas production in wheat straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS)[1]

Item		ATTD of TDF
$IVDMD^2_f$	WS	0.29
	SBH	-0.28
	Corn DDGS	0.98
	WS + SBH	0.99*
	SBH + corn DDGS	0.97*
	WS + corn DDGS	0.91*
	WS + SBH + corn DDGS	0.86*
A^3	WS	0.99
	SBH	0.59
	Corn DDGS	0.30
	WS + SBH	1.00*
	SBH + corn DDGS	0.97*
	WS + corn DDGS	0.91*
	WS + SBH + corn DDGS	0.91*

[1]Pearson correlation coefficients (r) are reported, there was 3 samples of WS, SBH, and corn DDGS separately.

[2]$IVDMD_f$: Large intestine fermentation disappearance.

[3]A: mL/g DM substrate, represents the asymptotic gas production.

*Means the $P \leq 0.05$.

Table 4.5. Prediction of the apparent total tract digestibility (ATTD) of total dietary fiber (TDF) of wheat straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS) from large intestine fermentation disappearance ($IVDMD_f$) and asymptotic gas production (A)[1]

ATTD of TDF	Equation[2]	*P* value	R^2	SE[3]	C(p)	Range of differences between predicted and measured ATTD of TDF, %
WS	(1) $y = 11.68 + 0.12 \times A$	0.08	0.98	0.46	NA	0.4-2.6
SBH	(2) NA[4]	NA	NA	NA	NA	NA
	(2)-1 $y = 13.80 + 3.26 \times B$[5]	0.10	0.97	0.82	NA	0.3-0.8
Corn DDGS	(3) $y = -199.83 + 3.49 \times IVDMD_f$	0.12	0.96	1.87	NA	0.3-4.2
WS + SBH	(4) $y = 5.01 + 0.18 \times A$	< 0.01	0.99	2.50	1.00	WS: 2.1-5.2; SBH: 1.0-5.5
SBH + corn DDGS	(5) $y = -120.87 + 2.36 \times IVDMD_f$	< 0.01	0.95	5.20	1.95	SBH: 3.5-9.6; corn DDGS: 3.6-7.0
WS + corn DDGS	(6) $y = 16.37 + 0.08 \times A$	0.01	0.82	4.75	1.07	WS: 0.5-5.8; corn DDGS: 0.2-16.9
WS + SBH + corn DDGS	(7) $y = -2.82 + 0.16 \times A$	< 0.01	0.82	10.68	3.13	WS: 32.8-45.7; SBH: 15.9-22.3; corn DDGS:5.1-27.6
	(7)-1 $y = -48.84 + 0.16 \times A + 28.05 \times C$[6]	< 0.01	0.97	4.79	1.10	WS: 0.7-4.2; SBH: 1.4-8.4; corn DDGS: 4.0-15.4

[1]A: mL/g DM substrate, represents the asymptotic gas production.
[2]There were 3 samples of WS, SBH, and corn DDGS separately, and there was no Mallows statistic (C(p)) in equation (3), probably because the sample size is too small.
[3]SE of the regression estimate defined as the root of the mean square error (MSE).
[4]Not applied.
[5]B: the time in hours after incubation at which half of the asymptotic amount of gas has been prodruced.
[6]C: a counstant that determines the sharpness of the switching characteristic of the gas profile.

Chapter 5. Prediction of digestible and metabolizable energy of corn distillers dried grains with solubles (DDGS) for growing pigs from *in vitro* digestible nutrients[1]

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ABSTRACT: Digestibility of nutrients (e.g. CP, NDF) varies among sources of corn distillers dried grains with solubles (DDGS). Therefore, prediction of DE and ME may improve when using DDGS specific equations that include digestible nutrients. The objective of this study was to develop prediction equations to estimate DE and ME content of DDGS using *in vitro* and *in vivo* values for digestible nutrients. Data from 12 sources of corn DDGS were obtained, including: chemical composition from wet lab analysis and near infrared spectroscopy (NIRS). *In vitro* DM digestibility (IVDMD) from gastric and small intestine ($IVDMD_h$), large intestine ($IVDMD_f$), and total tract ($IVDMD_t$) digestibility, were also determined along with *in vivo* determined apparent total tract digestibility (ATTD) of DM, CP, ether extract (EE), NDF, and ADF, and energy values (GE, DE, and ME). Correlation analysis was used to compare chemical composition from wet lab analysis with NIRS scans, and a stepwise selection of variables was performed using linear regression to establish DE and ME prediction equations. Results showed that NIRS did not correlate with wet lab analyzed chemical composition of corn DDGS. The DE ($P = 0.04$; $R^2 = 0.35$) and ME ($P = 0.04$; $R^2 = 0.52$) estimates of corn DDGS obtained from prediction equations had significant *P* values, but low R^2, when using wet lab analyzed chemical composition as inputs. Both significance and R^2 were poorer when using NIRS scanned chemical composition as inputs to predict DE ($P = 0.11$; $R^2 = 0.23$) and ME ($P = 0.11$; $R^2 = 0.24$). The DE ($P < 0.01$; $R^2 = 0.83$) and ME ($P < 0.01$; $R^2 = 0.76$) prediction equations: (5) DE = 854.5 + (3.6 × dig.DM) + (3.7 × dig.EE) + (2.0 × dig.NDF) and (6) ME = 704.5 + (3.3 × dig.DM) + (4.8 × dig.EE) + (2.6 × dig.NDF), had the best prediction when using *in vivo* digestible (dig.) DM, EE, and

fiber as inputs. *In vitro* digestible DM, but not *in vitro* digestible NDF, was selected for use in equations (7) DE = 6383.6 – (42.5 × IVdig.DM) + (35.4 × dig.EE) and (8) ME = 6635.1 – (49.8 × IVdig.DM) + (41.3 × dig.EE), but the significance and accuracy for both DE ($P = 0.07$; $R^2 = 0.45$) and ME ($P = 0.05$; $R^2 = 0.49$) was reduced. If chemical composition was further used to replace *in vivo* EE together with *in vitro* digestible NDF and DM, the prediction equations only used the concentration of ADF and EE as predictors instead of *in vitro* digestible NDF and DM. In conclusion, *in vivo* digestible NDF, DM, and EE are the best predictors for DE and ME content of corn DDGS fed to growing pigs. Using NIRS to determine chemical composition, *in vitro* digestible NDF, and *in vitro* digestible DM did not result in accurate predictions of DE and ME.

Key words: corn dried distillers' grains with solubles, digestible energy, metabolizable energy, *in vivo* and *in vitro* digestible nutrients, prediction equation

INTRODUCTION

Energy is the most expensive component in swine diets and an accurate supply of energy is, therefore, essential for optimizing pig production efficiency. Currently, the most accurate energy system is the NE system, which is widely used in Europe. The major NE systems used are all based on using digestible nutrients as inputs rather than chemical composition (Velayudhan et al., 2015). However, in the United States, commercial swine diets containing corn, soybean meal, and corn distillers dried grains with solubles (**DDGS**) are usually formulated based on the ME system because NE is more difficult and expensive to measure, and ME content of low fiber ingredients (corn and soybean meal) represents the true utilizable energy reasonably well. Corn DDGS is

one of the main ingredients used in U.S. swine diets, but it is highly variable in energy and nutrient content (Spiehs et al., 1999; Stein and Shurson, 2009). Furthermore, *in vivo* determinations of DE and ME of feed ingredients are time-comsuming and expensive. Consequently, *in vitro* methods have been developed to predict DM digestibility, and DE and ME content of feed ingredients (Boisen and Fernandez, 1997).

A three-step procedure has been used to predict DE and NE content of feed ingredients used in swine diets based on *in vitro* OM digestibility and chemical composition (Noblet and Jaguelin-Peyraud, 2007). A modified three-step procedure with the addition of measuring gas production, has also been used to predict *in vivo* DM, OM, and digestibility of NDF (Chen et al., 2014). However, no modified three-step procedure that includes measurement of gas production has been used to predict DE and ME of corn DDGS. Use of near infrared spectroscopy (**NIRS**) is another *in vitro* technology that has been used to predict chemical composition of corn DDGS (Zhou et al., 2012). As a result, it may be possible to obtain reliable estimates for digestible nutrients using NIRS predicted chemical composition along with *in vitro* digestibility of nutrients. Therefore, the objective of this study was to develop DE and ME prediction equations using either *in vivo* or *in vitro* digestible nutrients among sources of corn DDGS.

MATERIALS AND METHODS

Sample collection

We collected 12 corn DDGS samples used in our previous experiments (Kerr et al., 2013; Chapter 3) that contained values for *in vivo* apparent total tract digestibility of nutrients along with values of DE and ME estimated for growing pigs (Kerr et al., 2013).

These sources were obtained from different ethanol plants and represented diverse geographical locations of U.S. corn production, and different co-product processing technologies, to represent the variability in chemical composition among sources of corn DDGS available in the U.S. market.

Physicochemical analysis

Wet chemical analysis was determined using the following methods: DM (AOAC International, 2005; official method 934.01), EE (AOAC International, 2005; official method 920.39 (A)), NDF (Holst, 1973), ADF (AOAC International, 2005; official method 973.18), CP (AOAC International, 2005; official method 992.23).

NIR scan

The DM, CP, EE, starch, ADF, and NDF of 12 corn DDGS samples were scanned by DA 7250 NIR analyzer (Perten, Hägersten, Sweden) at the Crops Research Unit of the University of Minnesota (St. Paul, MN).

In vitro DM digestibility and asymptotic gas production

The *in vitro* DM digestibility from gastric and small intestine ($IVDMD_h$), large intestine ($IVDMD_f$), and total tract ($IVDMD_t$) were determined as described in Chapter 3.

Apparent total tract digestibility (ATTD) of nutrients

The ATTD of nutrients, as well as DE and ME (Table 5.1) of the 12 corn DDGS samples fed to growing gilts (BW 83.7 to 105.6 kg) were determined in previous *in vivo* animal experiments (Kerr et al., 2013).

Calculations

Digestible (Dig.) nutrients. Dig. nutrients, % = Nutrients, % × ATTD of nutrients [1]

In vitro digestible (IVdig.) nutrients. IVdig. Nutrients, % = Nutrients, % × *in vitro* nutrient digestibility · [2]

Statistical analyses

The PROC CORR of SAS (Version 9.3; SAS Inst. Inc., Cary, NC) was used to analyze the association between the chemical composition of corn DDGS samples based on wet lab analysis and NIR prediction. A value of $P < 0.05$ was considered as significant correlations. The PROC REG STEPWISE of SAS (Version 9.3; SAS Inst. Inc., Cary, NC) was used to select input variables for the equations to predict DE and ME content of corn DDGS samples from wet lab analyzed chemical composition or NIR predicted chemical composition; *in vivo* digestible nutrients; *in vitro* digestible nutrients with *in vivo* digestible nutrients; and *in vitro* digestible nutrients with chemical composition. Variance Inflation Factor (VIF) was used to determine multicollinearity, variables with VIF > 10 were considered as multicollinearity (Lamberson and Kaps, 2004), and removed from the prediction equations. The *P* value, R^2, and root of the mean square error (SEM) were used as parameters to determine the accuracy of the prediction equations.

RESULTS

Variability of composition and digestibility of nutrients

All comparisons in this section are numerical values and were not statistically analyzed. The GE (4,780 to 5,167 kcal/kg), DE (3,500 to 3,870 kcal/kg), and ME (3,266 to 3,696 kcal/kg) content of the 12 corn DDGS samples was variable (Table 5.1). The wet lab analyzed values of DM (88.8 to-90.0%) and CP (29.0 to 32.9%) were less variable

than energy values, while EE (4.9 to 13.2%), NDF (30.5 to 38.9%), and ADF (9 to 13.9%) content had greater variability than CP. The NIR predicted chemical composition also reflected similar variability in estimates compared with values derived from wet chemistry analysis for EE (7.1 to 14.4%), NDF (15.4 to 28.4%), and ADF (11.1 to 17.2%), compared with DM (89.9 to 91.7%) and CP (27.7 to 32.2%). The variability in *in vivo* nutrient digestibility showed similar trends. The ATTD of EE (65.7 to 81.2%), NDF (44.5 to 61.5%), and ADF (55.2 to 76.7%) were more variable than ATTD of DM (67.7 to 77.3%) and CP (78.0 to 84.8%). Furthermore, $IVDMD_f$ (44.3 to 64.2%) was more variable than $IVDMD_h$ (56.1 to 59.4%), and $IVDMD_t$ (77.2 to 83.5%) because $IVDMD_f$ is mainly a measure of fiber digestibility, and $IVDMD_h$ mainly represents CP, ether, and starch digestibility. Digestible nutrients were calculated based the chemical composition and digestibility of nutrients, resulting in digestible EE (3.2 to 9.1%), NDF (14.5 to 23.5%), ADF (5.5 to 10.4%) being more variable than digestible DM (60.9 to 68.6%) and CP (23.3 to 26.8%).

Correlation between chemical composition of wet lab analysis and NIR scan

No significant correlations were significant for DM, CP, starch, EE, ADF, and NDF concentration between wet lab analysis and NIR scans (Table 5.2). This indictating that the NIR scan was not an accurate method to predict chemical composition of corn DDGS using the limited number of samples evaluated in this study.

The DE and ME prediction equations predicted from chemical composition of wet lab analysis or NIR scan

For both DE and ME prediction equations (1) and (2), using chemical composition from wet lab analysis as the only variables in the equations resulted in significant *P* values ($P = 0.04$), but low R^2 (Table 5.3). Using NIR scanned chemical composition as the only variables in the equations did not have a significant *P* value ($P = 0.11$) and even lower R^2. These results suggest that chemical composition of this number of samples was not useful for accurately predicting DE and ME content, and also confirmed that NIR estimates were no better than wet lab analyzed chemical composition for use as predictors of DE and ME content.

The DE and ME prediction equations develped from digestible nutrients

As equations 5 and 6 demonstrate, *in vivo* digestible DM, NDF, and EE were good predictors of DE ($P < 0.01$; $R^2 = 0.83$) and ME ($P < 0.01$; $R^2 = 0.76$) content in DDGS (Table 5.4). *In vitro* digestible DM can also be selected to replace *in vivo* digestible DM as a predictor for DE (equation 7; $P = 0.07$; $R^2 = 0.45$) and ME (equation 8; $P = 0.05$, $R^2 = 0.49$) content. But both *in vivo* and *in vitro* digestible fiber were not selected for use in the prediction equations. The prediction accuracy of equations 7 and 8 was less than equations (5) and (6). When using chemical composition from wet lab analysis, along with *in vitro* digestible DM and fiber as candidate predictors, the stepwise procedure did not use *in vitro* digestible fiber or DM in the prediction (equations 9 and 10), and the prediction accuracy was relatively low ($P = 0.04$; $R^2 = 0.35$ for equation 9; $P = 0.05$, $R^2 = 0.33$ for equation 10). These results indicate that digestible EE is an important predictor of DE and ME content for corn DDGS.

DISCUSSION

The aim of this study was to investigate if commonly used *in vitro* methods could be used to accurately predict DE and ME content of corn DDGS. The use of digestible nutrients to predict energy, used in NE systems, is based on the notion that digestibility of nutrients accounts for the greatest differences among ingredients (Kil et al., 2013). *In vitro* ileal digestible CP and carbohydrates, along with calculated ileal digestible EE, are used as predictors in the Danish Potential Physiological Energy (PPE) system (Kil et al., 2013). In addition, using *in vitro* digestible nutrients as predictors of DE, ME, and NE can save time and cost compared with using traditional *in vivo* methodologies to determine digestible nutrients. Noblet and Jaguelin-Peyraud (2007) successfully used *in vitro* OM digestibility and chemical composition to predict DE and NE of feed ingredients, but Anderson et al. (2009) did not observe accurate prediction using *in vitro* OM digestibility as the only trait to predict DE and ME content of 20 corn by-products. However, no studies have been conducted to evaluate using *in vitro* DM digestibility or *in vitro* digestible DM to predict DE and ME content of a single feed ingredient, like corn DDGS.

The current study confirms that using *in vivo* digestible nutrients as predictors improves the accuracy for DE and ME prediction of corn DDGS. The prediction accuracy of the most accurate prediction equations (5 and 6) in the current study, was numerically comparable with that reported in a previous study (Noblet and Perez, 1993; Table 5.5). Compared with observed *in vivo* DE values, the difference between the predicted DE (kcal/kg) from equation 5 varied by about -405 kcal/kg (range from -483.9 to -321.8 kcal/kg, SD = 45.9, R^2 = 0.83); while the difference between predicted and *in vivo*

determined DE from Noblet and Perez (1993), varied by about 57.2 kcal/kg (range from -52.2 to 193.9 kcal/kg; SD = 84.9, R^2 = 0.89). Similarly, predicted ME compared with observed *in vivo* ME value differences using equation 6, varied by about 134.7 kcal/kg (range from -38.4 to 284.3 kcal/kg, SD = 106.1, R^2 = 0.76); while the difference between predicted and observed ME from Noblet and Perez (1993) varied about -370 kcal/kg (range from -469.5 to -279.8 kcal/kg; SD = 67.1, R^2 = 0.79).

However, *in vitro* digestible DM alone, or including *in vitro* digestible NDF or ADF, did not improve or have equivalent prediction accuracy of DE or ME for corn DDGS compared with *in vivo* digestible nutrients, based on comparing equations 5 and 6 with equations 7 and 8. Also, by comparing equations 9 and 10 with equations 1 and 2, we observed that using *in vitro* digestible DM and fiber as predictors did not improve DE and ME prediction precision compared with using chemical composition as predictor variables. The poor prediction from *in vitro* digestible DM might be explained by the poor correlation of $IVDMD_t$ with ATTD of DM (Chapter 3; *P* = 0.65; R^2 = 0.02), and low variability of digestible DM (SD = 2.40, CV = 4%) among the limited number of samples (n = 12) used in this study, because digestible DM should theoretically be strongly related to DE and ME content of corn DDGS. The explanation for the lack of correlation between *in vitro* and *in vivo* DM digestibility is not clear. According to Boisen and Fernández (1995), the prediction of apparent ileal digestibility of protein, by using an *in vitro* 2-step (pepsin and pancreatin digestion) procedure, had a very strong association (R^2 = 0.92). Therefore, we speculate that the difference may be related to lipid digestion because the emulsification of lipid is critical for *in vivo* lipid digestion and

it is difficult to mimic these conditions using the current three-step procedure. Also, the negative effects of *in vitro* digestible DM on DE and ME content (as indicated in equations 7 and 8), cannot be explained physiologically, suggesting that it may be just a calculation artifact because there was no correlation between *in vitro* and *in vivo* DM digestibility (Chapter 3; $P = 0.65$; $R^2 = 0.02$). Using *in vitro* digestible fiber to predict DE and ME may be difficult because *in vitro* fiber digestibility also had a low R^2 with ATTD of NDF ($P = 0.05$; $R^2 = 0.32$) and ATTD of ADF ($P = 0.01$, $R^2 = 0.47$), as discussed in Chapter 3, even though the *P* values were significant. These results indicate that *in vitro* digestible fiber is a good indicator of ATTD of NDF and ADF, but it appears to not be a good predictor with high precision in regression equations for estimating DE and ME content.

Our results show that digestible EE is an essential prediction variable for DE and ME equations for corn DDGS samples. However, *in vitro* lipid digestibility was not determined in the current study. *In vitro* lipid digestion has been commonly measured using the pH-stat method with pancreatic lipase in humans (Li et al., 2010; Li et al., 2011), but not in the three-step or modified three-step procedure. Further studies are need to determine *in vitro* lipid digestibility to improve the accuracy of prediction of DE and ME in feed ingredients, especially those like DDGS which contain a substantial amount of EE.

None of the equations selected *in vivo* digestible CP or CP concentration as predictors. This may be because of the relatively low variability in digestible CP (SD = 0.95, CV = 4%) among samples and the limited sample size (n = 12) used in this study

contributed to this result. Another limitation of the equations generated in this study, was the assumption that starch digestibility was 100% instead of measuring it directly. However, this assumption may be of minor importance because most of the starch in corn DDGS may be in the form of resistant starch, and the total concentration of starch in corn DDGS is relatively low (0.8 to 3.9%).

The utility of NIRS to predict DE and ME content of DDGS samples was the other *in vitro* method we evaluated. The NIRS has been used to predict DE and ME of corn DDGS (Zhou et al., 2012). Unfortunately, calibrations developed by Zhou et al. (2012) used DE and ME values that were calculated from ingredient chemical composition using equations derived for diets by Noblet and Perez (1993). The Noblet and Perez equations were specifically developed for diets and not for feed ingredients. The spectral model of NIRS also showed a higher prediction power for ME than using chemical components or *in vitro* digestible nutrients for wheat and barley fed to sheep (Deaville et al., 2009). However, the spectral method of NIRS requires a large number (> 200) of samples to develop robust calibrations, and the calculation process is very complicated. As a result, NIRS has not been used to predict DE and ME in corn DDGS fed to pigs yet. The use of NIRS to predict chemical composition is widely used in the feed industry as a faster and less expensive method to quantify nutrient content of feeds and feed ingredients compared with using wet lab analysis (Paulsen, 2010). Therefore, one of the aims of the current study was to attempt to use NIRS scanned chemical composition to replace wet lab analyzed chemical composition as an easier method to improve the DE and ME prediction. Data from current study did not show any correlation

of NIRS scanned chemical composition with wet lab analyzed values, and the regression equations for predicting DE and ME were not significant when using NIRS scanned chemical composition as predictor variables. We suspect that the database used for the NIRS calibration did not match the chemical composition of corn DDGS samples used in the study, and that the sample size used in this study was too small.

CONCLUSION

In conclusion, *in vivo* digestible fiber, DM, and EE are the best predictors for DE and ME of corn DDGS fed to growing pigs, whereas the NIRS determined values of chemical composition, *in vitro* digestible fiber, and *in vitro* digestible DM were not good predictors. *In vitro* digestible EE needs to be investigated as a potential predictor, and increasing the number of samples and their variability in chemical composition may improve the prediction accuracy of DE and ME content of corn DDGS fed to pigs.

Table 5.1. Chemical composition from wet lab analysis and near infrared spectroscopy (NIRS), *in vitro* DM digestibility (IVDMD), apparent total tract digestibility (ATTD) of nutrients, and digestible nutrients of 12 corn distillers dried grains with solubles (DDGS) samples

Measurement	Least	Mean	Greatest	SD	CV
Chemical composition (Kerr et al., 2013)					
GE, kcal/kg	4,780	4,966	5,167	114	2.3
DE, kcal/kg	3,500	3,638	3,870	132	3.6
ME, kcal/kg	3,266	3,417	3,696	147	4.3
DM, %	88.8	87.7	90.0	1.8	2.1
CP, %	29.0	30.6	32.9	1.2	3.8
Starch, %	0.8	2.2	3.9	1.1	48.9
EE, %	4.9	9.1	13.2	2.4	26.4
NDF, %	30.5	34.2	38.9	3.3	9.8
ADF, %	9.0	11.3	13.9	1.8	15.8
NIR predicted chemical composition, %					
DM, %	89.9	90.5	91.7	0.7	0.8
CP, %	27.7	29.6	32.2	1.4	4.7
EE, %	7.1	8.6	14.4	2.2	25.8
NDF, %	15.4	20.3	28.4	4.0	19.6
ADF, %	11.1	13.5	17.2	1.7	12.6
IVDMD and asymptotic gas production (Chapter 3)					
$IVDMD^{1}_{h}$, %	56.1	54.6	59.4	3.9	7.1
$IVDMD^{2}_{f}$,%	44.3	52.6	64.2	5.9	11.1
$IVDMD^{3}_{t}$,%	77.2	78.8	83.5	2.0	2.5
ATTD, % (Kerr et al., 2013)					
DM	67.7	71.2	77.3	2.7	3.8
CP	78.0	80.9	84.8	2.2	2.7
EE	65.7	63.9	81.2	8.5	13.3
NDF	44.5	53.4	61.5	5.2	9.7
ADF	55.2	67.3	76.7	6.8	10.0
Digestible nutrients[4], g/kg					
DM	601.0	626.7	686.0	25.1	4.0
CP	233.0	248.8	268.0	9.9	4.0
Starch[5]	8.0	22.4	39.0	11.0	48.9
EE	32.0	59.5	91.0	16.8	28.3
NDF	145.0	187.7	235.0	30.6	16.3
ADF	55.0	79.2	104.0	18.2	22.9

[1]$IVDMD_h$ = *In vitro* DM digestibility from simulated gastric and small intestinal hydrolysis.
[2]$IVDMD_f$ = *In vitro* DM digestibility from simulated large intestine fermentation.

[3]$IVDMD_t$ = *In vitro* DM digestibility from simulated total tract digestion.
[4]Calculated as chemical composition × ATTD of nutrients × 10.
[5]Assumed starch was 100% digested.

Table 5.2. Correlation of chemical composition of 12 corn distillers dried grains with solubles (DDGS) samples from wet lab analysis and near infrared spectroscopy (NIRS) values

Item	DM	CP	Starch	EE	ADF	NDF
r[1]	0.22	0.27	0.29	0.10	0.38	0.41
P-value	0.49	0.39	0.36	0.75	0.22	0.19

[1]r = Pearson's correlation coefficient.

Table 5.3. The DE and ME prediction equations from chemical composition using wet lab analysis or near infrared spectroscopy (NIRS) values of 12 corn distillers dried grains with solubles (DDGS) samples

Equation	***P***	**R^2**	**SE^2**
Wet lab chemical composition			
(1) DE = 4152.6 – (43.8 × ADF)	0.04	0.35	111.0
(2) ME = 3826.1 + (27.9 × EE)[1] – (57.5 × ADF)	0.04	0.52	112.9
NIR predicted chemical composition			
(3) DE = 4158.3 – (37.2 × ADF)	0.11	0.23	120.9
(4) ME = 3147.6 + (32.2 × EE)	0.11	0.24	134.6

[1]EE = ether extract.
[2]SE of the regression estimate defined as the root of the mean square error (MSE).

Table 5.4. Equations for DE and ME content predicted from digestible (dig.) nutrients, *in vitro* digestible nutrients, *in vivo* with digestible nutrients, or *in vitro* digestible nutrients with chemical composition of 12 corn distillers dried grains with solubles (DDGS) samples

Equation	***P***	**R^2**	**SE[3]**
Predicted from *in vivo* digestible nutrients			
(5) DE = 854.5 + (3.6 × dig.DM) + (3.7 × dig.EE)[1] + (2.0 × dig.NDF)	< 0.01	0.83	63.0
(6) ME = 704.5 + (3.3 × dig.DM) + (4.8 × dig.EE) + (2.6 × dig.NDF)	< 0.01	0.76	84.9
Predicted from IVDMD[2]			
(7) DE = 6383.6 – (4.2 × IVdig.DM) + (3.5 × dig.EE)	0.07	0.45	108.2
(8) ME = 6635.1 – (5.0 × IVdig.DM) + (4.1 × dig.EE)	0.05	0.49	116.2
Predicted from chemical composition and IVDMD			
(9) DE = 4152.6 – (43.8 × ADF)	0.04	0.35	111.0
(10) ME = 3969.1 + (27.9 × EE) – (57.5 × ADF)	0.04	0.52	112.9

[1]EE = ether extract.
[2]IVDMD = *in vitro* DM digestibility.
[3]SE of the regression estimate defined as the root of the mean square error (MSE).

Table 5.5. Comparison of differences of predicted DE and ME values, based on *in vivo* digestible (dig.) nutrients, with *in vivo* determined DE and ME values of 12 corn distillers dried grains with solubles (DDGS) samples

Equation	R^2	Least	Mean	Greatest	SD
Predicted from current study					
DE = 854.5 + (3.6 × dig.DM) + (3.7 × dig.EE)[1] + (2.0 × dig.NDF)	0.83	-483.9	-405.0	-321.8	45.9
ME = 704.5 + (3.3 × dig.DM) + (4.8 × dig.EE) + (2.6 × dig.NDF)	0.79	-38.4	134.7	284.3	106.1
Predicted from Noblet and Perez (1993)					
DE = (5.66 × dCP) + (9.37 × dEE) + (4.13 × ST) + (3.91 × SU) + (4.18 × dHemi) + (3.37 × dADF) + (4.24 × dRes)	0.89	-52.2	57.2	193.9	84.9
ME = (4.83 × dCP) + (9.58 × dEE) + (4.14 × ST) + (3.9 × SU) + (4.4 × dHemi) + (2.81 × dADF) + (4.04 × dRes)	0.79	-469.5	-370.7	-279.8	67.1

[1]EE = ether extract.

Chapter 6. Implication

Accurate swine diet formulation is essential for optimizing caloric and nutritional efficiency of pork production. The large variability in energy and nutrient composition among high fiber ingredients from different sources requires the development and use of relatively rapid, inexpensive, and accurate methods to determine dynamic estimates of these ingredients. The overall goal of this thesis was to develop a better understanding of the potential value of using a 3-step *in vitro* digestibility system to obtain dynamic estimates of energy and nutrient digestibility, and determine the physiological effects of feeding high fiber ingredients to pigs. Specifically, the results from these studies showed that corn DDGS, SBH, and WS 1) regulate intestinal cell differentiation differently, 2) have different fermentability, and 3) can be utilized by a modified three-step procedure and gas production technique to provide an accurate prediction of ATTD of TDF, and potentially, DE and ME content for growing pigs.

Chapter 2 described how feeding high amounts of theseingredients in growing pig diets favors the intestinal differentiation pathway that leads to secretory cells and decreases the absorptive cell lineage. Chapters 3 and 4 described how using a modified three-step procedure and gas production technique enables an accurate prediction of ATTD of TDF. Chapter 5 described the relative accuracy using modified three-step procedure to predict DE and ME content. However, identifying key potential predictors of *in vitro* digestible EE, along with analysis of a large number of samples are needed to improve prediction accuracy to achieve similar accuracy when using *in vivo* digestible nutrients as predictors. Therefore, although the use of the three-step *in vitro* procedure

appears to be useful, further refinements are needed to improve its practical application in evaluating high fiber ingredients.

Chapter 7. Appendix

7.1 Effects of growth stage of pigs on *in vitro* DM digestibility and *in vitro* gas production of 3 high fiber ingredients using modified three-step procedure[1]

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ABSTRACT: The current study was designed to compare *in vitro* fiber fermentation capacity of inocula from nursery and finishing pig. Three high fiber ingredients wheat straw (WS; 16 sources; NDF 69.0-83.4%), soybean hulls (SBH; 16 sources; NDF 60.9-67.7%) and corn dried distillers' grains with solubles (DDGS; 16 sources; NDF 28.8-44.0%) were evaluated. A modified three-step procedure, each 2 g sample was hydrolyzed for 2 h with pepsin and for a subsequent 4 h with pancreatin. Hydrolyzed residues were filtered, washed, dried, weighed, pooled within the same sample and used for subsequent fermentation using fecal inocula from nursery pigs at 5-7 wk of age and the same group of pigs at 19-21 wk of age. The volume of gas produced was recorded at 11 time points within 72 h of incubation. Gas production kinetics were fitted by non-linear model, *in vitro* DM digestibility and fitted gas production parameters were analyzed using a mixed model in SAS 9.3. Results showed differences of fiber digestibility in nursery and finishing pigs. The *in vitro* DM digestibility from large intestine ($IVDMD_f$) in nursery stage (74.7%) was greater ($P < 0.01$) than in finishing stage (55.0%); The asymptotic gas production (A, mL/DM substrate fermented) was also greater ($P < 0.01$) in nursery stage (206) than in finishing stage (185). The *in vitro* fiber digestibility of the 3 high fiber ingredients were also different. The $IVDMD_f$ in SBH diets (87.1%) was greater ($P < 0.01$) than corn DDGS diets (66.6%), which was greater ($P < 0.01$) than WS diets (40.9%). The asymptotic gas production of SBH (347) was also greater ($P < 0.01$) than corn DDGS (191), which was greater ($P < 0.01$) than WS (49). The interaction of pig growth stage and ingredient effect was also observed in the $IVDMD_f$ *and* asymptotic gas production ($P < 0.01$). In conclusion, the current modified three-step procedure showed nursery pigs have greater fiber digestibility than finishing

pigs in WS, SBH, and corn DDGS; fiber in SBH has the greatest digestibility, corn DDGS was intermediate, and WS was the lowest.

Key words: corn dried distillers' grains with solubles, growth stage, *in vitro* dry matter disappearance, soybean hulls, wheat straw

INTRODUCTION

High fiber ingredients have been used in swine diets for their competitive price and availability (Zijlstra and Beltranena, 2013), but variable nutrient and energy content and negative effects on digestion of protein, lipids, and starch are the main challenges. Corn distillers dried grains with solubles (**DDGS**) have variable total dietary fiber content (28.6-34.9%) and apparent total tract digestibility (**ATTD**; 29.3-57%; Urriola et al., 2013), which leads to variable ME content and variable digestible nutrients value of high fiber ingredients. Likewise, high content of total dietary fiber in corn DDGS decreased the proportion of energy utilized as metabolizable energy (GE:ME). Compared with corn, DDGS has less GE:ME (0.69 vs. 0.80%), making DDGS a less efficient source of energy.

Efficiency of energy utilization is also affected by age of pigs, especially the ability to degrade indigestible carbohydrates in the large intestine. Finishing pigs should have greater fiber fermentability than nursery pigs because enzymatic activity and capacity to secret enzymes increases with the pigs' age. Additinally, fermentative capacity of dietary fiber in the large intestine increases with age. A modified three-step *in vitro* procedure that involves pepsin and subsequent pancreatin hydrolysis followed by fecal fermentation has been developed and used to measure *in vitro* DM digestibility (**IVDMD**) and total gas production of various feed ingredients for swine (Bindelle et al., 2007a; Jha et al., 2015). Using this modified three-step procedure, studies found no

difference in *in vitro* DM disappearance between growing and finishing pigs (Bindelle et al., 2007). There was no difference of total gas production of sugar beet pulp fermented by pigs of 16-45 kg and 52-93 kg (Bindelle et al., 2007) either. However, there has been no research conducted to compare IVDMD and gas production among nursery and finishing pigs yet. Therefore, the objective of this study was to compare the differences of IVDMD and gas production of 3 high fiber ingredients in nursery and finishing pigs.

MATERIALS AND METHODS

Sample collection

A total of 48 samples were collected between May and July 2013; including 16 sources of wheta straw (WS), 16 sources of soybean hulls (SBH), and 16 sources of corn DDGS (Table 7.1.1). The WS samples were obtained from the University of Minnesota Beef Barn (St. Paul, MN), Nutrena (Minneapolis, MN), Dairyland Laboratories (Arcadia, WI), and the University of Minnesota West Central Research and Outreach Center (Morris, MN). The SBH samples were obtained from Nutrena (Minneapolis, MN), Archer Daniels Midland (Mankato, MN; Mexico, MO; Quincy, IL; Des Moines, IA; Fosteria, OH), AGP Ag Processing Inc. (Dawson, MN), Bunge (Council Bluffs, IA and Decatur, IN), and Consolidated Grain & Barge Soybean Processing (Mount Vernon, IN). Corn DDGS samples were obtained from a previous study (Kerr et al., 2013) and Highwater Ethanol (Lamberton, MN).

Enzymatic hydrolysis

All samples were ground to pass through a 1 mm mesh screen in a Wiley No. 4 Laboratory Mill (Arthur H. Thomas, Philadelphia, PA). The first 2-steps of the pepsin and pancreatin hydrolysis followed the steps developed by Boisen and Fernandez (1997),

subsequent steps followed modifications by Jha et al. (2011a, b). Briefly, 2 g of each sample (6 runs × 1 replicate per run) were weighed into a 500 mL conical flask and incubated at 39° C in a water bath. One hundred mL of phosphate buffer solution (0.1 *M*, KH_2PO_4: Na_2HPO_4 = 7:1, pH = 6.0) and 40 mL 0.2 *M* HCl solution (pH = 2.0) were added to each phase. The pH was adjusted to 2.0 by 1 *M* HCl or 1 *M* NaOH. Two mL of 5 mg/mL chloramphenicol (C0378; Sigma-Aldrich Corp., St. Louis, MO) solution (dissolved in ethanol) were added to prevent bacterial growth during hydrolysis. Then each replicate was treated with 4 mL of 100 mg/mL fresh porcine pepsin (P7000, 421 units/mg solids; Sigma-Aldrich Corp.) solution (dissolved in 0.2 *M* HCl) at 39° C and incubated in a water bath for 2 h, while all the flasks were shaken gently for 5 s by hand every 15 min. Afterward, 40 mL of 0.2 *M* phosphate buffer (KH_2PO_4: Na_2HPO_4 = 7:1, pH = 6.8) and 20 mL of 0.6 *M* NaOH were added to each flask. The pH was adjusted to 6.8 with 1 *M* HCl or 1 *M* NaOH and 4 mL of 100 mg/mL fresh porcine pancreatin (P1750, 4 × USP specifications; Sigma-Aldrich Corp.) solution (dissolved in 0.2 *M* phosphate buffer) was subsequently added. The hydrolysis continued for 4 h under the same conditions as pepsin hydrolysis.

After enzymatic hydrolysis, residues were collected by filtration (pore: 40 µm filter paper; VWR International, Radnor, PA), washed with enough distilled water to remove the residues from the wall of the serum bottles, ethanol (2 × 20 mL, 95%) and acetone (2 × 20 mL, 99.5%), dried for 72 h at 55° C, and weighed for determination of IVDMD. To obtain sufficient residues for the subsequent *in vitro* fermentation, 4-8 replicates of the enzymatic hydrolysis procedure were conducted depending on the amount of residues, the goal was to obtain enough residues for fermention (Table 7.1.2).

In vitro fermentation

The rate and amount of *in vitro* fermentation of the hydrolyzed residues was assessed by a cumulative gas production technique (Bindelle et al., 2007; Bindelle et al., 2009; Jha et al., 2015). Briefly, the hydrolyzed residues from enzymatic hydrolysis of the same sample were pooled for *in vitro* fermentation. Samples without addition of substrate (blank) were used as controls to adjust the values of fitted gas parameters. There were a total of 3 runs with 2 replicates per run of each blank, and hydrolyzed residues of WS, SBH, and corn DDGS (Table 7.1.2). About 0.2 g of each mixed hydrolyzed residue was weighed, all blanks and hydrolyzed residues of WS, SBH, and corn DDGS were incubated at 39^{o} C in a 125 mL rubber stoppered serum bottle with 30 mL buffer solution, including macro and micro-minerals (Menke and Steingass, 1988), and a fecal inoculum. Fecal inoculum was obtained from 5 pigs (Hampshire × Yorkshire) at nursery (5-7 wk) and finishing (19-21 wk) from University of Minnesota Saint Paul Campus Swine Research Facility (St. Paul, MN). Pigs had the same genetic background and were fed a standard commercial corn-soybean meal diet without antibiotics (Maverick Nutrition Inc., Austin, MN). Fecal samples were collected randomly from 3 out of the 5 pigs right after pigs defecated. Feces were immediately placed in Ziploc bags after collection, all air was removed, and bags were sealed and kept at 39^{o}C and delivered to the laboratory within 30 minutes. The inoculum was prepared by diluting blended feces in the inoculation solution that was composed of distilled water (474 mL/L), trace mineral solution (0.12 mL/L), *in vitro* buffer solution (237 mL/L), macro-mineral solution (237 mL/L) and resazarin (0.1% w/v solution; 1.22 mL/L) and filtered through folded cheesecloth. The final inoculum concentration was 0.05 g feces per mL of buffer. Each of

the 30 mL inoculum was transferred into bottles with the hydrolyzed residues and the bottles were sealed with rubber stoppers and placed in a water bath of 39^{o}C for incubation. Through the whole process, oxygen contact with the inoculum was avoided by adding reducing solution (distilled water 47.5 mL/L, 1 *M* NaOH 2 mL/L, Na_2S 335 mg/L) into the buffer solution and adding CO_2 (Jha et al., 2011a,b; Jha et al., 2015).

The gas produced during fermentation was measured at 2, 5, 8, 12, 16, 20, 24, 30, 36, 48 and 72 h through an inverted 25 mL burette with its stopcock end attached to vacuum and its open end submerged into a 39^{o} C water bath. Before assembling the burette apparatus, the headspace volume of the burette was determined. To measure gas volume at each time point, the inverted burette was filled with water to remove the air, then the serum bottle was quickly transferred from the incubation water bath to the water bath with the measuring burette, a 20-gauge needle was inserted through the rubber stopper. At each gas measurement time point, the operator opened the valve to release all of the gas into burette, and immediately recorded the volume displaced by the gas produced in the bottle using burette calibration marks. Once the measurement was recorded, the bottles were transferred back into the incubating water bath immediately. After *in vitro* fermentation, the residues were collected by filtration, washed, dried and weighed following the same procedures described for the hydrolyzed residues.

Physicochemical analysis

All the feed ingredient samples were analyzed at the University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO). Chemical analyses (Table 7.1.1) were performed according to standard AOAC (2006) procedures

using the following methods: DM (930.15), ADF (973.18), NDF (2002.04), and TDF (991.43).

Calculations

Hemicellulose. Hemicellulose, % = NDF, % - ADF, % [1]

Total feces needed to prepare the inoculum per run. Feces, g = 30 mL × No. of samples × No. of replicates per run × 0.05 g/mL [2]

Gas volume released at each time point. $V, mL = V_h + (25 – V_r)$, [3]

where, V_h is the volume of the burette headspace, V_r is the reading volume record, $V_h \leq V \leq V_h + 25$; 25 mL is the capacity of the burret; $V = V_r$, [4]

where, $0 < V < V_h$, V_r is measured the same as headspace volume;

$V = V_{r1} + V_{r2} + \ldots + V_{rn}$, [5]

where, $V > V_h + 25$, shut down the valve before gas went beyond the open end of the burette, recorded V_{r1}, and then repeated the procedure the second time and recorded V_{r2}, repeated the procedure n^{th} time until finish recording all the produced gas.

In vitro dry matter digestibility from large intestine fermentation (**$IVDMD_f$**) The $IVDMD_f$ was calculated as follows: $IVDMD_f$, % = [(dry weight of hydrolyzed residues – dry weight of the residues after fermentation)/ dry weight of hydrolyzed residues] × 100[6]

Kinetics of gas production. Gas accumulation curves recorded during the 72 h of fermentation were modified according to monophasic model from Groot et al., (1996):

$$G = A / (1 + (B^C/t^C)), \quad [7]$$

where G (mL/g DM substrate) denotes the amount of gas produced per g of DM incubated, A (mL/g DM) represents the asymptotic gas production, B (h) is the time after

incubation at which half of the asymptotic amount of gas has been formed, C is a constant determining the sharpness of the switching characteristic of the profile.

When calculating $IVDMD_f$ and accumulated gas production volume, all data were corrected by subtracting blank values at each stage from observed values.

Statistical analyses

The kinetics of gas production parameters were modeled using PROC NLIN procedure of SAS 9.3 (SAS Inst. Inc., Cary, NC). The $IVDMD_f$ and fitted gas production kinetic parameters were analyzed using the PROC MIXED procedure of SAS 9.3. The growth stage (nursery and finishing) and ingredient (WS, SBH, and corn DDGS) were fixed factors, sources of ingredients, run, and replicates were random factors. Differences were considered significant when $P \leq 0.05$ and a trend when $0.05 < P < 0.1$.

RESULTS

Effect of different growth stage of pigs on *in vitro* fiber digestibility

Results showed that the *in vitro* DM digestibility from large intestine was greater ($P < 0.01$) in nursery stage (74.7%) than in finishing stage (55.0%; Table 7.1.4). The asymptotic gas production (A, mL/DM substrate fermented) was also greater ($P < 0.01$) in nursery stage (206) than in finishing stage (185). The time reaching half of the asymptotic gas production (B, h) was slower ($P < 0.01$) in nursery stage (35.9) than in finishing stage (20.5). The constant C that determines the sharpness of the switching characteristic of the profile was smaller ($P < 0.01$) in nursery stage (1.86) than in finishing stage (1.90).

Effect of different high fiber ingredient on *in vitro* fiber digestibility

The *in vitro* DM digestibility from large intestine in SBH diets (87.1%) was greater ($P < 0.01$) than corn DDGS diets (66.6%), which was greater ($P < 0.01$) than WS diets (40.9%). The asymptotic gas production of SBH (347) was greater ($P < 0.01$) than corn DDGS (191), which was greater ($P < 0.01$) than WS (49). The time reaching half of the asymptotic gas production (B, h) of SBH (32.9) was slower ($P < 0.01$) than WS (26.9), which was slower ($P < 0.01$) than corn DDGS (24.8). The constant C that determines the sharpness of the switching characteristic of the profile of WS (2.61) was greater ($P < 0.01$) than SBH (1.93), which was greater than corn DDGS (1.11).

Interaction of growth stage and ingredient effects on *in vitro* fiber digestibility

Interactions between the growth stage effects and the ingredient effects ($P < 0.01$) were also observed in the *in vitro* DM digestibility, asymptotic gas production, time reaching half of the asymptotic gas production, and the constant C. The $IVDMD_f$ was not different if inoculated with feces from nursery pigs or finishing pigs. However, $IVDMD_f$ of DDGS or SBH with feces from nursery pigs was greater than when inoculated with feces of finishing pigs. Likewise, asymptotic gas production of WS was not modified by addition of inocula from nursery or finisher pigs.

DISCUSSION

Overall, in both nursery and finishing pigs, the comparison of fermentability among WS, SBH, and corn DDGS confirmed the results we obersved from Chapters 3 and 4: SBH > corn DDGS > WS. However, the greater fiber fermentability observed in nursery pigs compared to finishing pigs is beyond expectation. Finishing pigs should have greater fiber fermentability than nursery pigs because enzymatic activity and capacity to secret enzymes increases with the pigs' age. In another study, there was no

difference of total gas production of sugar beet pulp fermented by pigs of 16-45 kg and 52-93 kg (Bindelle et al., 2007). However, there was no published data for the comparaison of nursery pigs and finishing pigs regarding the effect on total gas production. Unfortunately, we are not able to explain the data at this moment.

CONCLUSION

Use of the current modified three-step procedure showed that nursery pigs had greater fiber digestibility than finishing pigs for WS, SBH, and corn DDGS, which is inconsistent with several published in vivo studies showing that nursery pigs digest fiber to a lesser extent that finishing pigs. Fiber in SBH had the greatest digestibility, corn DDGS was intermediate, and WS was the lowest.

Table 7.1.1 Analyzed composition of wheat straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS), DM basis

Item	WS[1]	SBH[1]	Corn DDGS[1]
Total dietary fiber	90.8	78.9	34.4
NDF	76.5	65.5	34.3
NDF/TDF	84.1	83.0	100.6
ADF	54.9	49.6	11.2
Hemicellulose[2]	21.6	15.9	23.1

[1]There are 16 sources each for WS, SBH, and corn DDGS.
[2]Calculated as NDF - ADF.

Table 7.1.2 The number of runs and replicates in the enzymatic hydrolysis step for wheat straw (WS), soybean hulls (SBH), corn distillers dried grains with solubles (DDGS), and blanks

Item	2-step enzymatic hydrolysis		Fermentation	
	Run[1]	Replicates per run	Run	Replicates per run
WS	4-6	1	3	2
SBH	5-6	1	3	2
DDGS	6-8	1	3	2
Blank			3	2

[1]The number of runs was determined by the amount of residues of each sample, the goal was to obtain sufficient residue for fermentation.

Table 7.1.3 *In vitro* dry matter digestibility from large intestine ($IVDMD_f$) and fitted kinetic parameters on the gas accumulation recorded for wheat straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS) at different growth stage of swine corrected by blank[1]

Item	Effects	IVDMDf, %	A[2]	B[3]	C[4]
Nursery	WS	40.3	42	31.1	3.37
	SBH	105.4	401	51.3	1.32
	Corn DDGS	78.5	173	25.3	0.91
Finishing	WS	41.5	54	22.6	1.88
	SBH	68.8	293	14.5	2.54
	Corn DDGS	54.7	209	24.3	1.29
Growth stage	Nursery	74.7	206	35.9	1.86
	Finishing	55	185	20.5	1.9
Ingredient	WS	40.9	49	26.9	2.61
	SBH	87.1	347	32.9	1.93
	Corn DDGS	66.6	191	24.8	1.11
P value	Growth stage	< 0.01	< 0.01	< 0.01	0.68
	Ingredient	< 0.01	< 0.01	< 0.01	< 0.01
	Growth stage × Ingredient	< 0.01	< 0.01	< 0.01	< 0.01
Pooled SE		3.9	15.7	2.5	0.14

[1]There are 16 sources of WS, SBH, and corn DDGS separately.
[2]A: mL/g DM substrate, represents the asymptotic gas production.
[3]B: h, the time after incubation at which half of the asymptotic amount of gas has been formed.
[4]C: A constant determines the sharpness of the switching characteristic of the profile.

7.2 Effects of different dietary fiber diets on apparent total tract digestibility of nutrients, energy values, nitrogen retention, and carcass yield of growing pigs[1]

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ABSTRACT: This experiment was designed to test the effects of different dietary fiber diets on apparent total tract digestibility (ATTD) of nutrient, ATTD of GE, and carcass yield of growing pigs. The experiment used 34 barrows and 2 gilts (BW 84 ± 7 kg) with a changeover design to test ATTD of 9 treatments: 3 wheat straw (WS) diets, 3 soybean hulls (SBH) diets, and 3 corn distillers dried grains with solubles (DDGS) diets with balanced total dietary fiber (TDF) concentration (22.3%). There were 2 consecutive 13-d periods with 10 d adaptation and 3 d collection of each period, 0.5% TiO_2 was used in diets as indigestible marker. Pigs had free access to water, but limited amount of feed (2.5% of initial BW of each period). The comparisons among WS, SBH, and corn DDGS diets and the comparisons within different sources of WS, SBH, and corn DDGS diets were analyzed with ingredients (n = 3) and ingredient sources (n = 3) nested under each ingredient type as fixed factors and period as a random factor. The results showed that among WS, SBH, and corn DDGS diets, SBH diets had the greatest ($P < 0.01$) ATTD of GE (88.0%), DM (88.8%), and total dietary fiber (TDF; 78.9%); corn DDGS diets had the greatest ($P < 0.01$) ATTD of CP (85.1%), and WS diets had the greatest ($P < 0.01$) ATTD of ether extract (EE; 79.0%). Differences of ATTD of GE and nutrient were also observed within sources of WS, SBH, and corn DDGS diets. The WS diets (3,175 kcal/kg) had lower ($P < 0.01$) DE value than SBH diets (3,610 kcal/kg) and corn DDGS diets (3,552 kcal/kg). The ME value of WS diets (3,098 kcal/kg) was lower ($P < 0.01$) than corn DDGS diets (3,361 kcal/kg), which were lower ($P < 0.01$) than SBH diets (3,516 kcal/kg). The nitrogen retention of corn DDGS diets (41.9%) was lower ($P < 0.01$) than SBH diets (58.4%) diets and WS diets (59.3%). No statistical differences of DE, ME, or nitrogen retention were observed among sources of WS, SBH, and corn DDGS

diets. No differences of harvest BW, carcass weight or carcass yield among or within sources of WS, SBH, and corn DDGS diets were observed. However, the relative weight of liver to BW of corn DDGS diets (1.51%) was also greater than WS diets (1.40%) and SBH diets (1.38%) and SBH5 diet was greater ($P < 0.05$) than SBH4 diet. In conclusion, different commercial high fiber diets containing WS, SBH, and corn DDGS have different ATTD of energy and nutrients and different sources within WS, SBH, and corn DDGS also have different ATTD of energy and nutrients. However, no differences were observed of carcass yield either among or within sources of WS, SBH, and corn DDGS diets.

Key words: wheat straw, soybean hulls, corn distillers dried grains with solubles, apparent total tract digestibility, energy

INTRODUCTION

The negative effect on energy and nutrient utilization of high fiber swine diets has been tested in both commercial high fiber diets such as distillers dried grains with solubles (**DDGS**; Kim et al., 2013), sugar beet pulp (Noblet and Le Goff et al., 2001), wheat bran (Dégen et al., 2009; Freire et al., 1998; Noblet and Le Goff et al., 2001), soybean hulls (**SBH**; Mroz et al., 1996; Noblet and Le Goff et al., 2001), oat by-products (Bach Knudsen and Hansen, 1991); and also diets composed of pure fiber sources like pectin (Mosenthin et al., 1994), guar gum (Owusu-Asiedu et al., 2006), cellulose (Mroz et al., 1996; Owusu-Asiedu et al., 2006). The other negative effect of high fiber diets fed to swine is the increased the weight of gastrointestinal tract (**GIT**; Kass et al., 1980) and reduced carcass yield (Asmus et al., 1994). Most research was conducted with a balanced CP, ether extract (**EE**), and energy value to study the specific effects of different fiber

inclusion, solubility, or viscosity on energy and nutrient utilization (Dégen et al., 2007) or carcass yield (Asmus et al., 1994). However, there were few studies to formulate more practical commercial diets with balanced dietary fiber concentration but not energy and other nutrients. There is also a lack of research studies to investigate the effects of different sources within the same high fiber ingredient on energy and nutrient utilization as well as carcass yield. Therefore, the objectives of this study were 1) to estimate the effects of different commercial high fiber diets on apparent total tract digestibility (**ATTD**) of energy and nutrients, as well as carcass yield; 2) to estimate the effects of different sources within high fiber ingredients on ATTD of energy and nutrients, as well as carcass yield.

MATERIALS AND METHODS

All experimental procedures involving animals were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Sample collection and diet formulation

We collected 3 sources of each of 3 different high fiber feed ingredients: wheat straw (WS), SBH, and DDGS with an expected range of chemical composition and nutritional characteristics. We considered each sample as a different source. The WS sources were collected from the University of Minnesota St. Paul Campus Beef Barn (St. Paul, MN), UMore Park (Rosemount, MN), and Southern Research and Outreach Center (Waseca, MN). The SBH sources were obtained from Archer Daniels Midland at 3 different locations (Mexico, MO; Des Moines, IA; Valdosta, GA). The DDGS sources were from Heron Lake BioEnergy, LLC (Heron Lake, MN), Big River Resources (Dyersville, IA), and Commonwealth Agri Energy (Hopkinsville, KY). The chemical

compositions of the sources are present in Table 7.2.1.

The composition of experimental diets is showed in Table 7.2.2. The WS, SBH, or DDGS was the only source of fiber in each diet. The diets were formulated in accordance to NRC (2012) to contain about 22% TDF. Titanium dioxide (TiO_2) was added 0.5% as an indigestible marker.

Animal experiment

Thirty-four growing barrows and 2 gilts (BW 84 ± 7 kg, Large White × Danish Landrace) were housed in the metabolic unit at the Southern Research and Outreach Center in Waseca, MN. Barrows and gilts were individually housed in metabolism cages and allotted to 4 blocks with 9 pigs in each block. The experimental design used was a changeover design. The 9 growing pigs from each block were fed the 9 different experimental diets in 2 consecutive 13-d periods. Each period consisted of a 10-d adaptation period and a 3-d collection period. In each adaptation period, the pigs were gradually adapted to the cages and fed the experimental diets.

Pigs were provided feed twice a day (0800 and 1600) at the calculated amount equivalent to 2.5% of their body weight. Water was available *ad libitum* in nipple drinkers. Pigs were weighed at the beginning and at the end of each period, before the morning meal. Feces and urine of each pig were collected separately twice a day at 0800 and 1600 for 3 d at each collection period. A window screen and a funnel were placed under the cage, a bucket with 30 mL 6 N HCl was used to collect urine under the funnel. The HCl was added to the urine to prevent N losses by evaporation of ammonia. Around 200 g of feces per day were collected in sealable plastic bags and kept frozen in coolers until further processing. At the conclusion of all collection periods, fecal samples were

weighed and oven dried at 60°C for 4 d, ground through a 1-mm screen, and subsampled for storage and shipment to the Midwest Laboratories (Omaha, NE) for further analysis. The urine was weighed by scale and measured the volume by graduate cylinders. A total of 5% volume of each urine collection was taken and the accumulation of all the samples for each period was stored in -20°C for further analysis.

At the end of the experiment, pigs were weighed after overnight fasting and then harvested at the Andrew Boss Meat Science Laboratory of the Unviersity of Minnesota (Saint Paul. MN). Liver weight and carcass weight were recorded.

Physicochemical analysis

All the sources were analyzed in Midwest Laboratories (Omaha, NE). The analysis methods for ingredient sources, diets, feces, and urine were as follows: DM (method 930.15, AOAC 2006), GE (ASTM D 5865-13), CP (method 992.15, AOAC 2006), ether extract with acid hydrolysis (EE) (method 922.06, AOAC 2006), ADF (Ankom Technology), NDF (Ankom Technology), total dietary fiber (TDF) (method 991.43), lignin (method 973.18, AOAC 2006), titanium (WDXRF), bulk density (USP <616> method I), viscosity (Perten, AACC international).

Calculations

Hemicellulose. Hemicellulose, % = NDF, % - ADF, % [1]

Cellulose. Cellulose, % = ADF, % - Lignin, % [2]

Apparent total tract digestibility of the diet nutrients. ATTD, % = [(Nutrient in ingredient/TiO_2 in ingredient - Nutrient in feces/ TiO_2 in feces)/ (Nutrient in ingredient/ TiO_2 in ingredient)] × 100 [3]

Digestible energy of the diets. DE, kcal/kg = GE, kcal/kg × ATTD of GE, % [4]

Metabolizable energy of the diets. ME, kcal/kg = DE, kcal/kg – Total energy in urine, kcal/kg [5]

Nitrogen retention, % = (Nitrogen intake, g/d – nitrogen output in feces, g/d – nitrogen output in urine, g/d)/(nitrogen intake, g/d) [6]

Statistical analysis.

The ATTD of nutrients, energy values, and N retention were analyzed using the PROC MIXED procedure of SAS (Version 9.3; SAS Inst. Inc.) with the ingredients (n = 3), and sources (n = 3) nested under each ingredient as fixed factors and period as a random factor.

RESULTS

Apparent total tract digestibility of nutrients.

Statistical differences of ATTD of GE, DM, CP, EE, and TDF were observed among WS, SBH, and corn DDGS diets (Table 7.2.3). The ATTD of GE, DM, and TDF of SBH diets (88.0%, 88.8%, and 78.9% respectively) were greater ($P < 0.01$) than that of corn DDGS diets (79.4%, 80.4%, and 43.0% respectively), which were greater ($P < 0.01$) than those of WS diets (75.9%, 75.8%, and 26.7% respectively). The ATTD of CP of corn DDGS diets (85.1%) was greater ($P < 0.01$) than that of WS diets (81.4%), which was greater ($P < 0.01$) than SBH diets (77.6%). The ATTD of EE of WS diets (79.0%) was greater ($P < 0.01$) than that of SBH diets (68.3%), which was greater ($P < 0.01$) than that of corn DDGS diets (59.0%).

There were differences ($P < 0.05$) within sources of WS, SBH, and corn DDGS diets. Within sources of WS diets, WS1 and WS3 diets had greater ($P < 0.01$) ATTD of GE and ATTD of EE than WS2 diet; WS3 diet had greater ($P < 0.05$) ATTD of DM than

WS1 diet and WS2 diet. Within sources of SBH diets, SBH4 diet had greater ($P < 0.01$) ATTD of CP and ATTD of EE than SBH5 diet and SBH6 diet. Within sources of corn DDGS diets, corn DDGS8 diet had greater ($P < 0.01$) ATTD of TDF than corn DDGS9 diet (36.0%).

Energy value and nitrogen retention of high fiber diets fed to swine.

Statistical differences ($P < 0.01$) of DE and ME among WS, SBH, and corn DDGS diets fed to swine were observed. The WS diets (3,175 kcal/kg) had lower ($P < 0.01$) DE value than SBH diets (3,610 kcal/kg) and corn DDGS diets (3,552 kcal/kg). The ME value of WS diets (3,098 kcal/kg) was lower ($P < 0.01$) than corn DDGS diets (3,361 kcal/kg), which were lower ($P < 0.01$) than SBH diets (3516 kcal/kg). The nitrogen retention of corn DDGS diets (41.9%) was lower ($P < 0.01$) than SBH diets (58.4%) diets and WS diets (59.3%).

No statistical differences of DE, ME, or nitrogen retention were observed among sources of WS, SBH, and corn DDGS diets.

Relative liver weight and carcass yield of high fiber diets fed to swine.

No differences of harvest BW, carcass weight or carcass yield among WS, SBH, and corn DDGS diets were observed (Table 7.2.5). However, the weight of liver of pigs fed with corn DDGS diets (1,537 g) were greater ($P < 0.01$) than pigs fed with WS diets (1385 g) and SBH diets (1,370 g); the relative weight of liver to BW of pigs fed with corn DDGS diets (1.51%) was also greater than pigs fed with WS diets (1.40%) and SBH diets (1.38%).

No differences of BW, liver weight, carcass weight, and carcass yield were observed within sources of WS, SBH, and corn DDGS diets. The relative liver weight to

BW were not different within sources of WS diets and corn DDGS diets either, but SBH5 diet was greater ($P < 0.05$) than SBH4 diet.

CONCLUSION

Results from this experiment suggest that different commercial high fiber diets containing WS, SBH, and corn DDGS have different ATTD of energy and nutrients. Likewise, different sources within WS, SBH, and corn DDGS also have different ATTD of energy and nutrients. There were no differences in carcass yield either among or within sources of WS, SBH, and corn DDGS.

Table 7.2.1. Analyzed composition of wheat straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS), DM basis

Fiber source	GE, kcal/kg	CP, %	EE[1], %	ADF, %	NDF, %	TDF[2], %	Lignin, %	Hemicellulose[3], %	Cellulose[4], %
WS									
1	4,050	4.1	2.2	53.0	79.1	77.2	6.6	26.0	46.4
2	4,160	6.2	2.5	53.0	80.0	82.6	7.6	27.0	45.4
3	4,032	3.8	2.8	52.7	78.2	80.0	7.5	25.5	45.2
SBH									
4	4,070	11.5	2.4	51.0	68.4	80.2	3.5	17.4	47.5
5	4,065	12.6	2.8	48.2	63.2	75.9	3.9	15.1	44.3
6	3,998	11.3	2.8	50.2	66.4	75.9	4.9	16.2	45.2
DDGS									
7	4,879	29.9	9.8	13.8	32.7	38.2	1.8	18.8	12.0
8	4,842	31.6	9.0	17.4	35.1	37.5	4.6	17.7	12.8
9	4,776	30.7	8.2	14.5	33.8	37.8	2.6	19.2	11.9

[1]Ether extract with acid hydrolysis.
[2]Total dietary fiber.
[3]Calculated as NDF-ADF.
[4]Calculated as ADF-lignin.

Table 7.2.2 Composition and nutrient concentration of wheat straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS) diets

Item	WS			SBH			DDGS		
	1	**2**	**3**	4	5	6	7	8	9
Ingredient, %									
WS	23.00	23.00	23.00	0.00	0.00	0.00	0.00	0.00	0.00
SBH	0.00	0.00	0.00	30.00	30.00	30.0	0.00	0.00	0.00
Corn DDGS	0.00	0.00	0.00	0.00	0.00	0.00	55.00	55.00	55.00
Plasma spray-dried	4.73	4.73	4.73	4.73	4.73	4.73	4.73	4.73	4.73
Corn starch	61.10	61.10	61.10	56.66	56.66	56.66	34.73	34.73	34.73
Casein	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Fish meal, menhaden	6.74	6.74	6.74	3.77	3.77	3.77	0.00	0.00	0.00
Titanium dioxide	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Dicalcium phosphate 18.5%	0.00	0.00	0.00	0.30	0.30	0.30	0.00	0.00	0.00
Limestone	0.32	0.32	0.32	0.44	0.44	0.44	1.44	1.44	1.44
Sodium chloride	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Grow-finishing vitamin and mineral premix[1]	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Total	99.99	99.99	99.99	100.00	100.00	100.00	100.00	100.00	100.00
Analyzed nutrient composition, DM basis									
GE, kcal/kg	4,160	4,169	4,174	4,106	4,105	4,099	4,517	4,480	4,429
CP, %	13.0	13.6	12.4	13.2	13.7	13.1	22.2	24.0	23.0
EE[2], %	2.9	2.6	3.1	2.4	2.5	2.3	6.3	6.3	6.0
ADF, %	12.4	12.5	13.0	15.3	13.9	14.7	8.6	10.0	7.7
NDF, %	22.2	23.5	26.9	21.3	19.8	23.5	18.7	19.6	20.4
Total dietary fiber, %	23.0	23.4	21.4	24.7	23.2	25.0	20.7	20.9	18.3
Lignin, %	2.2	2.3	2.2	0.8	0.9	1.5	2.0	2.8	2.1
Titanium, %	0.33	0.33	0.30	0.27	0.33	0.28	0.35	0.38	0.37
Bulk density, g/100cm^3	0.37	0.42	0.40	0.64	0.61	0.60	0.67	0.68	0.67
Viscosity, centipoise	1,989	2,142	2,455	532	607	817	311	301	310

[1]The vitamin and trace mineral premix (ANS Swine G-F premix) provided the following (per kg of diet): vitamin A 3,527,392 I.U., vitamin D 3 661,386 I.U., vitamin E 13,228 I.U., vitamin K (MPB) 1,323 mg, riboflavin 2,205 mg, niacin 13,228 mg, pantothenic acid 8,818 mg, vitamin B12 13 mg, Iodine (EDDI) 119 mg, selenium (Selenite) 119 mg, SQM organic zinc 22,046 mg, SQM organic iron 13,228 mg, SQM organic manganese 454 mg, SQM organic copper 1,543 mg.

[2]Ether extract with acid hydrolysis.

Table 7.2.3. Effects of wheat straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS) diets on apparent total tract digestibility (ATTD) of nutrients

Item	GE, %	DM, %	CP, %	EE[1], %	TDF[2], %
WS	75.9[c]	75.8[c]	81.4[b]	79.0[a]	26.7[c]
SBH	88.0[a]	88.8[a]	77.6[c]	68.3[b]	78.9[a]
Corn DDGS	79.4[b]	80.4[b]	85.1[a]	59.0[c]	43.0[b]
SEM	0.5	0.5	0.5	0.9	1.6
P value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
WS					
1	77.1[A]	75.2[B]	81.2	80.4[A]	24.2
2	72.9[B]	74.3[B]	81.2	74.7[B]	29.4
3	77.8[A]	78.0[A]	81.9	81.9[A]	26.4
SEM	1.0	0.9	0.7	1.4	3.0
P value	< 0.01	0.02	0.70	< 0.01	0.52
SBH					
4	88.1	88.8	79.4[A]	73.2[A]	79.0
5	87.0	87.8	76.4[B]	65.1[B]	75.0
6	88.9	89.7	77.1[B]	66.5[B]	82.7
SEM	0.9	0.9	0.7	1.4	2.6
P value	0.37	0.30	< 0.01	< 0.01	0.12
Corn DDGS					
7	79.3	80.2	85.2	58.9	43.1[AB]
8	79.6	80.8	85.8	58.2	49.8[A]
9	79.2	80.3	84.4	59.8	36.0[B]
SEM	0.9	0.9	0.7	1.4	2.6
P-value	0.95	0.88	0.30	0.72	< 0.01

[1]Ether extract with acid hydrolysis.

[2]Total dietary fiber.

[a,b,c]Expressed statistic differences between different ingredients ($P < 0.05$).

[A,B,C]Expressed statistic differences between different sources nested under each ingredient ($P < 0.05$).

Table 7.2.4 Effects of wheat straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS) diets on energy value and nitrogen retention

Item	DE, kcal/kg	ME, kcal/kg	Nitrogen retention, %
WS	3,175[a]	3,098[a]	59.3[b]
SBH	3,610[b]	3,516[c]	58.4[b]
Corn DDGS	3,552[b]	3,361[b]	41.9[a]
SEM	24	25	1.4
P-value	< 0.01	< 0.01	< 0.01
WS			
1	3,207	3,147	64.1[A]
2	3,072	2,990	56.6[B]
3	3,245	3,158	57.0[B]
SEM	43	45	2.4
P value	0.03	0.03	0.05
SBH			
4	3,618	3,518	60.6
5	3,570	3,465	56.3
6	3,642	3,566	58.1
SEM	40	41	2.3
P value	0.43	0.24	0.43
Corn DDGS			
7	3,582	3,383	39.8
8	3,566	3,375	44.0
9	3,507	3,324	41.9
SEM	40	41	2.4
P value	0.38	0.56	0.48

[a,b,c] Expressed statistic differences between different ingredients ($P < 0.05$).

[A,B,C] Expressed statistic differences between different sources nested under each ingredient ($P < 0.05$).

Table 7.2.5 Effects of wheat straw (WS), soybean hulls (SBH), and corn distillers dried grains with solubles (DDGS) diets on relative liver weight and carcass yield

Item	N	BW, kg	Liver, g	Carcass, kg	Liver: BW, %	Carcass yield, %
WS	11	97.9	1,385[b]	70.0	1.40[b]	72.3
SBH	12	97.9	1,370[b]	70.8	1.38[b]	73.1
Corn DDGS	11	100.5	1,537[a]	73.9	1.51[a]	74.2
SEM		3.2	42	3.1	0.04	0.9
P value		0.72	0.02	0.4	0.04	0.11
WS						
1	4	95.7	1,353	69.3	1.40	73.2
2	3	99.2	1,482	71.2	1.47	72.3
3	4	98.7	1,319	69.6	1.33	71.3
SEM		4.9	75	4.3	0.06	1.3
P value		0.84	0.31	0.93	0.30	0.43
SBH						
4	4	99.3	1,305	71.7	1.28[B]	73.1
5	4	97.0	1,485	71.0	1.53[A]	74.1
6	4	97.6	1,320	69.7	1.35[AB]	71.9
SEM		4.8	71	4.2	0.06	1.3
P value		0.93	0.16	0.92	0.02	0.31
Corn DDGS						
7	4	99.3	1,531	72.2	1.53	73.6
8	3	104.4	1,589	76.9	1.50	74.1
9	4	97.9	1,490	72.6	1.50	74.9
SEM		4.9	75	4.3	0.06	1.3
P-value		0.60	0.66	0.65	0.94	0.67

[a,b,c]Expressed statistic differences between different ingredients ($P < 0.05$).

[A,B,C]Expressed statistic differences between different sources nested under each ingredient ($P < 0.05$).

7.3 Modified three-step *in vitro* procedure

The objective of this section is to descript procedures of characterization of dietary fiber fermentation characteristics that were used in previous chapters.

Part 1-Enzymatic Hydrolysis

Material and methods:

Feedstuffs:

Feedstuffs (WS, SBH, and corn DDGS, 3 samples of each ingredient) should be ground using a laboratory mill equipped with a 1 mm screen.

Reagents:

▪ Phosphate buffer 0.1M, pH 6.0 (Buffer 1)

- Prepare a KH_2PO_4 0.1 M (1.3609 g/100 mL, 9.5263 g/700 mL) solution
- Prepare a Na_2HPO_4 0.1 M (1.4196 g/100 mL) solution
- Mix 175 ml KH_2PO_4 0.1 M with 25 ml Na_2HPO_4 0.1 M (KH_2PO_4: Na_2HPO_4 = 7:1)

▪ Phosphate buffer 0.2 M, pH 6.8 (Buffer 2)

- Prepare a KH_2PO_4 0.2 M (2.7218 g/100 mL) solution
- Prepare a Na_2HPO_4 0.2 M (2.8392 g/100 mL) solution
- Mix 100 ml KH_2PO_4 0.2 M with 100 ml Na_2HPO_4 0.2 M (KH_2PO_4: Na_2HPO_4 = 1:1)

▪ HCl 0.2 M

- 16.72 ml HCl 37 % / 1 L

▪ HCl 1 M

- 8.36 ml HCl 37 % / 100 ml

▪ NaOH 1 M

- 4 g NaOH / 100 ml

▪ NaOH 0.6 M

- 24 g NaOH / 1 liter

▪ Ethanol 95 %

▪ Acetone 99.5 %

▪ Chloramphenicol solution

- 0.5 g of chloramphenicol (Sigma n°C-0378) in 100 ml ethanol

▪ Pepsin solution (use always fresh solution)

- Dissolve 400 mg of porcine pepsin (2000 FIP-U g^{-1}, Merck n°7190 OR 421 units/mg solids, 1:10 000, Sigma P7000) in 4 ml dHCl 0.2M –for each sample (! CAUTION: Pepsin is irreversibly inactivated when pH > 6). Warm solution to 37 C and take off heat and put onto another cool stir pad. Temperatures over 38.6 C will deactivate the enzyme thus reducing digestion. Beware that suppliers provide multiple types of products with variable concentration and activity of enzymes.

▪ Pancreatin solution (use always fresh solution)

- Dissolve 400 mg of porcine pancreatin (4 × USP specifications, Sigma n°P-1750) in 4 ml phosphate buffer 0.2 M, pH 6.8-for each sample. Warm solution to 37 C and take off heat and put onto another cool stir pad. Temperatures over 38.6 C will deactivate the enzyme thus reducing digestion.

Material: Making solutions uses volumetric flasks and graduated cylinders

- 500 ml conical flasks (36)
- Ankom bags (pore size 50 µm)
- Stirrer and magnets
- Disposable transfer pipets

- 100, 50 and 25 ml graduated cylinders
- Filtration crucibles (6)
- Vacuum device
- Water-bath with gentle agitation (39°C)
- pH-meters (2)
- Dispensers (2),
- Timers (1)

Operating mode

DAY 1 (weigh sample for hydrolysis and DM)

- Weigh samples (36)
  - ✓ Use big size weigh paper
  - ✓ Change different weigh papers between different samples
  - ✓ All the samples weigh between 1.9900-2.0099 g
  - ✓ Make sure put all the samples into the bottom of the flasks, not stick to the wall of the flasks
  - ✓ Put each of the sample into specific number of flask
  - ✓ After weigh all the samples, put stopper to avoid dust
- DM (9 × 2 replicates)
  - ✓ In the beginning, use 2-3 small desiccators and 4 beakers to warm the scale
  - ✓ Weigh hot beakers (36 × 2 replicates), whenever the scale is cooling down, it is needed to be rewarming before weighing any hot beakers
  - ✓ After finishing weighing hot beakers, wait the scale to cool down and then weigh samples 0.9900 – 1.0099 g (36 × 2 replicates)

- ✓ Weigh all the samples to specific number of beakers

- Prepare all the solutions to small containers, set up in the water bathes to be warm up the next day
- Dry new bags (36)

DAY 2

- Weigh hot beakers again and calculate DM of the samples

DAY 3 (Hydrolysis)

STEP 1 (6:30 am-7:00 am)

- ✓ Warm up the water bathes to 39°C, warm up buffer 1 and dH_2O
- ✓ Keep very low level of water in the water bathes and set up 16 flasks (with sample) into small water bath, 32 flasks into bigger water bath, make sure the flasks do **NOT** float
- ✓ Weigh pepsin 0.4 g/4 ml HCl/sample, prepare 1.5 times amount (batch 1)

STEP 2 (7:00 am-10:00 am)

- 7:00 - 8:00 am (Prepare pepsin hydrolysis)

➢ **Prepare for Pepsin Hydrolysis:**

- ✓ Resolve pepsin
- ✓ Serve dispenser
- ✓ Use small funnel with long tube to add 100 ml of phosphate buffer 1 (0.1M, pH 6.0) to the flask gently and mix gently to make sure that no dry sample sticks to the wall and bottom of the flask
- ✓ Add 40 ml HCl (0.2M) to the flask gently and mix gently to make sure that no dry sample sticks to the wall and bottom of the flask

- ✓ Adjust pH to 2 using 1 M HCl or NaOH and transfer pipets, rinse the electrode with dH_2O above the flask; Erin: help record pH; Pedro: help cover the stoppers

- 8:00 am

  - ➢ **Pepsin Hydrolysis:**

  - ✓ Helper 1 adds pepsin solution (start shaking timer) and 2 ml of the Chloramphenicol solution using syringe

  - ✓ Help cover the flask

  - ✓ Put more warm dH2O to smaller water bath till the water level is a little bit higher than the flask water level

  - ✓ Warm buffer 1, weigh pepsin 0.4 g/4 ml HCl/sample, prepare 1.5 times amount as needed

- 8:00 – 10:00 am

  - ➢ **Pepsin Hydrolysis:**

  - ✓ Shake every 15 min

- 9:00 -10:00 am

  - ➢ **Prepare for Pancreatin Hydrolysis:**

  - ✓ Warm buffer 2

  - ✓ Weigh pancreatin 0.4 g/4 ml buffer 0.2 M, prepare 1.5 times amount as needed

STEP 3 (10:00 – 15:00)

- 10:00-11:00 am

  - ➢ **Prepare for Pancreatin Hydrolysis:**

  - ✓ Resolve pancreatin

  - ✓ Serve dispenser

- ✓ Use small funnel with long tube to add 40 ml of phosphate buffer 2 (0.2M, pH 6.8) to the flask gently and mix gently to make sure that no dry sample sticks to the wall and bottom of the flask
- ✓ Add 20 ml NaOH (0.6M) to the flask gently and mix gently to make sure that no dry sample sticks to the wall and bottom of the flask
- ✓ Adjust pH to 6.8 using 1 M HCl or NaOH and transfer pipets, rinse the electrode with dH_2O above the flask; Pedro: help record pH; Erin help cover the stoppers

- 11:00 am
  - ➢ **Pancreatin Hydrolysis:**
  - ✓ Adds 4 ml/each pancreatin solution (start shaking **timer**) using syringe
  - ✓ Help cover the flask
  - ✓ Put more warm dH2O to bigger water bath till the water level is a little bit higher than the flask water level
  - ✓ 11:00 – 15:00
  - ➢ **Pancreatin Hydrolysis:**
  - ✓ Assistant: shake every 15 min
- 15:00
  - ➢ **Prepare for Filtration:**
  - ✓ Turn off smaller water bath and put all the bottles out of the water bath, put the rubber band in the bottom of Ankom bags

STEP 4 (15:00 -17:00)

- 15:00 -17: 00
  - ➢ **Filtration:**

- ✓ Filtration every 6/time, make sure keep all the vacuum at the same rate
- ✓ Serve bags, trays, clicks, dH_2O, ethanol and acetone
- ✓ Washing glass wares

- 17:00-17:30
  - ➢ Put all the wet bags under the fans for overnight

DAY 4

- Seal bags, grind residues by hand and weigh bags
- Put the bags into 50 °C oven

DAY 5

- Weigh bags under in 50 °C oven

DAY 6

- Weigh bags under in 50 °C oven

After Day 6

- Statistical Analysis
- Mix the hydrolyzed residues from different runs together to represent large intestine fermentation

Note: DM disappearance during hydrolysis of common feedstuffs can vary between 0.15 and 0.70. It is recommended to run enough hydrolysis to recover 4 g of hydrolyzed residue for subsequent fermentation and additional analyses (NSP, starch, protein, etc.) on the residue.

Part 2- *In Vitro* Fermentation (Gas production)

Materials Needed:

Feces collection

5 Grower Pigs (First Batch: 5-7 weeks old, Second Batch 17-20 weeks old)

Ziploc bags

Thermos with warm water around 39 centigrade

Scale (range > 300 g)

Gloves, boots and overalls

Fermentation

125 mL serum bottles

5 ml pipette

10 ml peptide and peptide aid

Small beaker

Volumetric Flask (1 L)

4L Erlenmeyer flask

Stirrer and magnets

Cheesecloth

Blender

Reagent Preparation: *(Stock solutions of the first three solutions can be prepared ahead of time and will remain stable for up to a year)*

a. *In vitro* rumen buffer solution (1 liter): Dissolve the following (in order) in dH_20

Ammonium bicarbonate – 4.0 g/L

Sodium bicarbonate – 35 g/L

b. *In vitro* macromineral solution (1 liter): Dissolve the following (in order) in dH_20

Sodium phosphate dibasic, anhydrous **–** 5.7 g/L

Potassium phosphate monobasic, anhydrous – 6.2 g/L

Magnesium sulfate, heptahydrate – 0.583 g/L

Sodium chloride – 2.22 g/L

c. *In vitro* micromineral solution (for 100 ml): Dissolve the following (in order) in 10 ml conc. HCl, then bring to volume to 100ml with dH_20 (carefully!)

Calcium chloride, dehydrate – 13.2 g

Manganese (III) chloride, tetrahydrate – 10 g

Colbalt chloride, hexahydrate – 1 g

Ferric chloride, hexahydrate – 8 g

Make the following solutions fresh on the day of Inoculation

d. Day of Inoculation Solution (For 1 L Solution) Dissolve the following in order:

dH_2O -474 mL

Trace Mineral Solution- 0.12 mL

In Vitro Buffer Solution- 237 mL

Macromineral Solution- 237 mL

Resazarin (0.1% w/v solution) - 1.22 mL

e. Reducing Solution Dissolve the following in order:

dH_2O- 47.5 mL

1M Sodium Hydroxide- 2 mL

Sodium Sulfide, nonahydrate - 335 mg

Fecal Collection:

a. Fill a thermos to bring with to the swine barn, fill 39 °C warm water into the thermos

b. Collected approximately 200 g feces from 5 grower pigs

c. Preventing as much oxygen exposure as possible, place the feces into a plastic Ziploc bag. Make sure that the bag is sealed tightly.

d. After feces are collected place the sealed bag into the thermos filled with warm water for transport back to the laboratory

e. Mix the feces by hand in the bag so that it is a uniform mixture of the feces from all of the pigs

Preparation of the flasks and medium:

a. Homogenize the residues of each particular substrate prepared during enzymatic hydrolysis

b. Weigh 0.2 grams of the residues and place into 125 mL serum bottle. Make sure to include at least two blanks that contain medium and inoculum without substrate.

c. Prepare the "Day of Inoculation" solution by adding in order: Trace Mineral solution, *In Vitro* Buffer, Macromineral solution, and resazurin.

d. Prepare the reducing solution and add to the medium. Bubble the solution with CO_2 to displace any oxygen. Resazuring will indicate the reduction state of the solution. A pink or purple color indicates oxidation, while yellow or colorless indicates reduction.

Addition of feces and inoculation of the flasks:

a. Make sure inoculation solution is fully reduced.

b. Add ~400 mL of solution to the collected feces and blend using a Waring blender to suspend the fiber-associated bacteria in the liquid. Blending should be done under constant gassing of CO_2

c. Filter the solution through 4 layers of cheese cloth under vacuum. The funnel should be covered with a plastic sheet and the feces/buffer slurry should be gassed with CO_2 while filtering. Figure 6.2 shows a diagram of the blender and the filtering apparatus
d. Complete the solution with the reduced buffer in order to reach a dilution of 0.05 g of feces per mL of buffer.
e. Still gassing the feces/buffer slurry with CO_2, use a bottle-top dispenser to fill each serum bottle with 30mL of slurry. Gas the inoculated serum bottle with CO_2 for ten seconds to prevent oxygen inclusion before sealing with butyl rubber cap and crimp top. Place serum bottles directly into 39 °C water bath
f. Measure the gas production at 2, 5, 8, 12, 16, 20, 24, 30, 36, 48, and 72 hours.

Measurement of gas from the serum bottles

a. Before assembling burette apparatus, the headspace in a 250 ml burette is determined by weighing the amount of dH_20 required to fill the burette to the first calibration mark when mounted right side up. This weight will be added to each volume.
b. Prepare apparatus for gas measurement as shown in figure 6.3 below. The 25 ml burette is inverted, with the stopcock end attached to vacuum (with trap) to ease the filling after each measurement. The open end of the burette is submersed into a bath of warm water. A short length of tubing is fixed with a valve and 20 gauge needle assembly on one end. The other end of the tube is inserted into the open end of the burette.
c. The water in the bath should be maintained at 37-40° C in water bath. Immediately before each measurement, fill burette to ‘top’ with warm bath water by opening stopcock with vacuum applied.

d. To measure volume at each time point: The serum bottle is quickly transferred to the warm bath with burette (filled). Water in the bath should cover bottle up to crimp seal on bottle. The needle, with valve closed, is inserted through stopper. Slowly open the valve to release gas into burette. Wait for bubbles to stop. Immediately record volume using burette calibration marks.
e. Replace bottle into incubation bath. Multiple measurements can be taken on each flask; however, integrity of the seal will deteriorate over time, reducing gas holding ability. Recommend use of small gauge needle, and possibly changing top quickly (to avoid O_2 inclusion) after gas measurement.
f. pH should be measured at the end of the experiment in order to ensure proper buffering of fermentation acids. pH should remain above 6)
g. Remaining substrate can be used in the Van Soest procedures

Residue filtration and drying (the same as hydrolysis filtration and drying)

a. Set up nylon bags (Ankom, pore size) or filter paper (VWR, pore size : 40 µm)
b. Turn on vacuum
c. Filter
d. Wash twice with 95% ethanol twice with 99.5% acetone
e. Seal with rubber band (for nylon bags) or pin
f. Dry in 60 °C oven

Summary of pepsin utilization in selected *in vitro* procedures used to measure digestibility of nutrients in diets and ingredients for growing pigs

Authors, year	Substrate, g	Pepsin	Enzyme activity[1]	Suppliers assay units	Total pepsin, mg	Enzyme: substrate		Conversion[2],	Supplier assay
						mg/g	activity U/g		
Merk products in EU									
Boisen, 1991	1.0	Merck 7190	2000	FIP U/g	10	10	20	60000	Merck 7190, 2000 FIP = 1: 3,000 1:10,000
Boisen, 1995	1.0	Merck 7190	2000	FIP U/g	10	10	20	60000	
Boisen, 1997	0.5	Merck 7190	2000	FIP U/g	25	50	100	300000	
Bindlle, 2007	2.0	Merck 7190	2000	FIP U/g	100	50	100	300000	
		Sigma 7000	385	U/mg solids	100				
Sigma products in USA									
Jha, 2011	2.0	Sigma P0609	30	U/mg solids	80	40	1.2	12000	
Jha, 2011	2.0	Sigma P0609	30	U/mg solids	80	40	1.2	12000	
Jaworski, 2012	0.5	Sigma P7000	250	U/mg solids	25	50	12.5	125000	
Regmi, 2009	1.0	Sigma P7000	800-2500	U/mg protein	10	10	25	250000	Sigma P 7000 was = 1: 10,000
			250	U/mg solids	10	10	2.5	25000	
Regmi, 2009	0.5	Sigma P7000	800-2500	U/mg protein	25	50	125	1250000	
			250	U/mg solids	25	50	12.5	125000	
Anderson 2012	0.5	Sigma P7012	2500	U/mg protein	25	50	125	1250000	

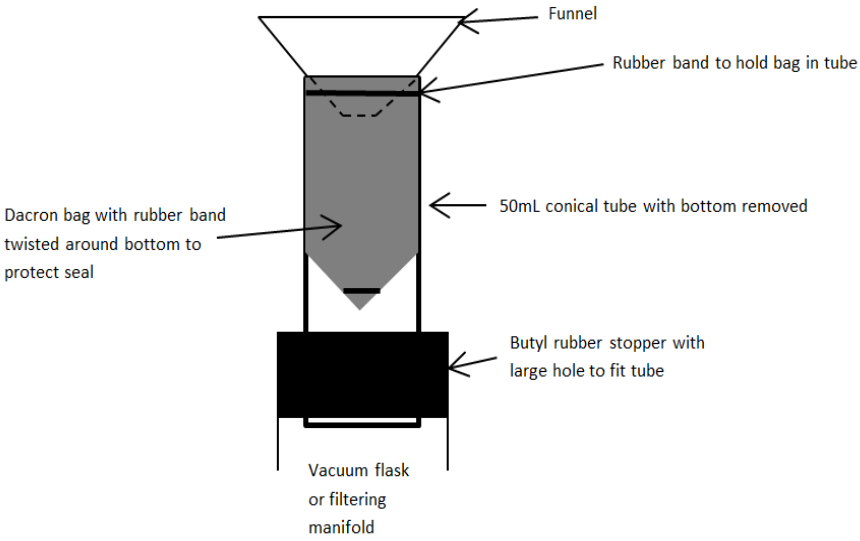
[1]Assays to determine enzyme activity are not standard among suppliers. Therefore, units vary. Sigma has used 2 different assays to measure enzyme activity. These are U/mg protein or U/mg of solids

[2]Conversion from 2,000 FIP to 1: 10,000 AOAC 971.09

Summary of pancreatin utilization in selected *in vitro* procedures used to measure digestibility of nutrients in diets and ingredients for growing pigs

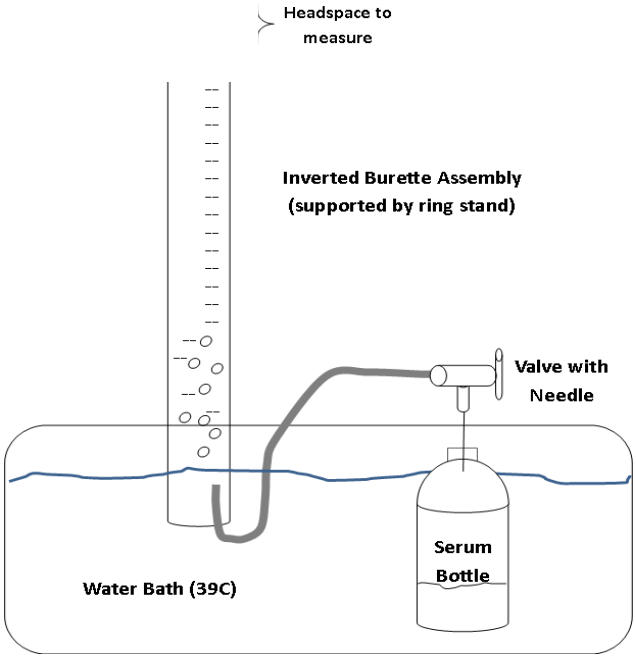
Authors, year	Substrate, g	Pancreatin	Enzyme activity[1]	Suppliers assay units	Total pepsin, mg	Enzyme: substrate	
						mg/g	activity U/g
Moughan, 1989	1.0	Merck	30,000 lipases, 1,400 proteases, 30,000 amylases	FIP U/g	4	4	120 lipases, 5.6 protease, 120 amylases
Boisen, 1991	1.0	Sigma P1790	-	-	50	50	-
Boisen, 1995	1.0	Sigma P1790	-	-	50	50	-
Boisen, 1997	0.5	Sigma P1750	Right now: 4	USP specifications	100	200	-
Bindle, 2007	2.0	Sigma P1750	Right now: 4	USP specifications	200	100	-
Jha, 2011	2.0	Sigma P1750	Right now: 4	USP specifications	200	100	-
Jha, 2011	2.0	Sigma P1750	Right now: 4	USP specifications	200	100	-
Regmi, 2009	1.0	Sigma P1750	20.8 lipase, 110 proteases, 113 amylases	U/mg solids	150	150	3.12 lipase, 16.5 protease, 16.95 amylase
Regmi, 2009	0.5	Sigma P1750	20.8 lipase, 110 proteases, 113 amylases	U/mg solids	100	200	3.12 lipase, 16.5 protease, 16.95 amylase

[1]Assays to determine enzyme activity are not standard among suppliers. Therefore, units vary. Sigma has used 2 different assays to measure enzyme activity. These are U/mg protein or U/mg of solids



Arangement of filtering setup for drolysis. This can be set up on ıltiple filtering flasks or on a ınifold with multiple ports to filter veral bags simultaneously.

Arrangement of inverted burette assembly for gas measurement rumen contents.



7.4 Barn protocol for metabolism experiment of Chapter 4

Calculate parameters of diets used in Chapter 4

Items	Amount needed
Average daily feed intake + waste, kg/d	3.8
Pig No. total	36
No. of test ingredient	3
Pig No. of each ingredient diet	12
No. of specific diet for each ingredient	3
Pig No. of each specific diet	4
Experiment period, d	28
No. of total diets	9
Total amount of each ingredient diet, kg	1276.8
Total amount of each specific diet, kg	425.6, consider as 500

Supplies used in the barn

Items	Source
Daily use in the barn	
1. Metabolic crates, collection funnels, window screen	Waseca
2. Barn Scale to weigh pigs	Waseca
3. Buckets to collect urine	Waseca
4. Tools for repairing	Waseca
5. IACUC printed version	
6. Scale to weigh daily feed and urine, 2 are better	St. Paul lab
7. Tapes, rainbow colors are better	St. Paul lab
8. Marker, sharpie and pens	St. Paul lab
9. Scissor	St. Paul lab
10. Gloves	St. Paul lab
11. Mask	St. Paul lab
Feces and urine collection	
12. Whirl-pack bags or Ziploc bags	St. Paul lab
13. Graduate cylinders to collect urine-4 L, 2 L, 1 L, 500 mL, 100 mL	St. Paul lab
14. Funnels	St. Paul lab
15. Cheesecloth	St. Paul lab
16. Urine storage bottles, 1 L or 500 mL	St. Paul lab
17. Coolers-at least 3 big ones	St. Paul lab
18. Dry ice and liquid nitrogen	St. Paul lab
19. HCl,37% 2-3 bottles (4 L size)	St. Paul lab
20. Dispenser (50 mL)	St. Paul lab
21. Tools like iron strings and aluminum containers to block the waste water from urine	St. Paul lab
22. Metal screen to put on top of urine buckets	Waseca

Ingredient suppliers for diet formulation

Ingredient, lbs	Quantity	Company	Address	Contact information
Corn DDGS	606.3	Heron Lake BioEnergy, LLC	91246 390th Avenue, Heron Lake, MN 56137	507-793-0077
	606.3	Big river Resources, LLC	3294 Vine Road, Dyersville, IA 52040	800-769-1066, CHS
	606.3	Commonwealth Agri-Energy	P.O. Box 766, 4895 Pembroke Road, Hopkinsville, Kentucky 42240	Land'olakeL/800-333-9774
Soybean hulls	330.7	ADM	400 E Holt St, Mexico, MO 65265	800-336-2326
	330.7	ADM	1935 E Euclid Ave, Des Moines, IA 50313	515-263-2147
	330.7	ADM	1841 Clay Rd, Valdosta, GA 31601	229-242-0100
Wheat straw	253.5	Campus dairy barn	St. Paul, MN 55108	612-624-3062
	253.5	UMore Park	15003 Akron Ave, Rosemount, MN 55068	
	253.5	Waseca	35838 120th St, Waseca, MN 56093	
Plasma spray-dried	469.3	Waseca		
Fish meal, menhaden	347.6	Waseca		
Dicalcium phosphate, 18.5%	9.9	Waseca		
Limestone	72.8	Waseca		
Salt	29.8	Waseca		
Grow-finishing Vitamin Premix	14.9	Waseca		
Trace Mineral Premix	14.9	Waseca		
Corn Starch	5042.7	John Goihl		jgoihl@aol.com
Milk, Casein	297.6	John Goihl		jgoihl@aol.com
TiO_2	49.6	Dr. Kerr/ Chicago Sweeteners		

7.5 Pig harvest protocol

Samples

Sample-from Waseca

1. Blood samples: 2000 rpm, 15 min -need 36 tubes for blood collection, and at least 72 tubes for centrifuged serum, leave the samples at RT for at least 30 min before centrifugation. Collected at fed status: 1 ml, 1 tube for each sample.
2. Feces: snap freeze in liquid nitrogen, transport by dry ice and storage in -80 °C-need 72 1.5 ml centrifuge tubes, collect tiny little.
3. Urine: snap freeze in liquid nitrogen, transport by dry ice and storage in -80 °C -need 36 1.5 ml centrifuge tubes, collected 1 mL.

Sample-from Campus: Snap freeze, -80C

1. Blood: fast status (DPP4), 5 ml tube, plasma, centrifuge right after sample collection
2. Stomach: antrum and corpus
3. Intestine: duodenum, ileum, colon

Supplies

Supplies	Source	Amount
Liquid N	Montgomery	1 tank
Dry ice	U Market	50 lb
Regular ice	St. Paul lab	1 box
Cooler	Diagnostic lab	2
1.5 mL centrifuge tube	St. Paul lab	1 pack (500)
Scalpel blades	Lab	102
Scale	Waseca	1
Sharps container	Lab	102
Syringe and needle	Lab	34
Forceps	St. Paul lab	4

Transportation

1. A day before harvest: 7:00 am, start feeding pigs, 8: 00 am, start bleeding pigs (fed

status) and 12:00 pm, feed again. Then start to weigh and load pigs to the truck. Around 2:30 pm, pigs leave Waseca and arrive beef barn in campus around 4:00 pm. The pigs will be fasted since 12:00 pm (Monday); the fasted blood will get on campus. Erin and Milena will set up collection supplies and tools at the meats laboratory

2. Pigs will stay in beef barn and delivered twice before harvest. The first batch of pigs in pig barn will deliver to the meat lab at 5:30 am, pig harvest start at 6: 00 am. Then second batch maybe around 8-9:00 am.

Pigs Harvest

1. A day before harvest
   a) Nate: (4:00 pm) get ready for pigs in beef barn: water and pens
   b) 2 helpers: set up the collection table, pasted IDs
2. The day of harvest (start at 5:30 am in meat lab): sample collection
   a) Helper 1: moves pigs from pen to scale, record live weight (dressing percentage), move pig into place
   b) Meat lab: electric shock, bleeding
   c) Helper 2: collect blood samples
   d) Meat lab: moves pigs to scalding tank
   e) Meat lab: clean carcass, hang, and open the carcass, remove the entire digestive track with bladder into a clean container, move clean container away from the carcass and to the collection table
   f) 3 helpers (gut table): stomach, duodenum, ileum, colon, cecum content, urine
   g) Meat lab: final rinse and collect empty carcass weight

Note:

1. Record time of stick and time of last tissue collected
2. After heads are removed then the ear and ear tag stays with the viscera – transfer to the inspector table

7.6 Ki-67 staining using rabbit specific HRP (horseradish peroxidase)/DAB (3,3'-diaminobenzidine) detection kit for rabbit specific HRP/DAB (ABC) detection IHC kit (abcam®) and counting procedure

Staining procedure:

1. Deparaffinize and rehydrate formalin-fixed paraffin-embedded tissue section.

2. Antigen retrieval. Slides are covered by 10 mM sodium citrate (pH = 6.0) were boiled for 30 minutes, cool down and wash 3 times in Tris-buffered saline with 1% Triton (TBS-T) buffer.

3. Add enough drops of Hydrogen Peroxide Block to cover the sections. Incubate for 10 minutes. Wash 4 times in buffer.

4. Apply non-specific binding block and incubate for 5 minutes at room temperature, rinse 1 time in buffer.

5. Apply primary antibody (concentrated and pre-diluted Rabbit Monoclonal Antibody were diluted 1:200 by Van Gogh Yellow; Biocare Medical Inc) and incubate 2 hours.

6. Wash 4 times in buffer. Apply biotinylated goat anti rabbit IgG(H+L) and incubate for 10 minutes at room temperature. Wash 4 times in buffer.

7. Apply Streptavidin Peroxidase and incubate for 10 minutes at room temperature.

8. Rinse 4 times in buffer. Add 20 µl DAB Chromogen to 1 ml of DAB Substrate, mix by swirling and apply to tissue. Incubate for 10 minutes. Rinse 4 times in buffer.

9. Add enough drops of Hematoxylin to cover the section. Incubate for 1 minute.

10. Rinse 10 times in tap water.

11. Merge the section to distilled water.

12. Counterstain tissue sections: 75% ethanol, 75% ethanol, 100% ethanol, 100% ethanol,

50% ethanol+50%xylene, xylene, xylene, xylene for 5 mins each.

13. Add mounting medium to cover the section and dry the slides under the hood.

Counting procedure:

1. Put the slides under the microscope (Olympus BX53).

2. Randomly choose 10 fields under × 200 magnitude and count the Ki-67 positive cells (brown marked cells) portion in well oriented crypts within each field.

7.7 Alcian Blue / PAS staining (newcomer supply) and counting procedure

Staining procedure:

1. Deparaffinize sections thoroughly in three changes of xylene, 3 minutes each. Hydrate through two changes each of 100% and 95% ethyl alcohols, 10 dips each. Wash well with distilled water.

2. Place slides in Solution A, Acetic Acid, 3% Aqueous Solution for 3 minutes.

3. Place slides directly into Solution B: Alcian Blue Stain Solution, 1%, pH 2.5 for 30 minutes.

4. Wash slides in gently running tap water for 10 minutes; rinse in distilled water.

5. Place slides in Solution C: Periodic Acid, 0.5% Aqueous Solution for 10 minutes.

6. Wash slides in running tap water for 5 minutes; rinse in distilled water.

7. Place slides in Solution D: Schiff Reagent, McManus for 20 minutes.

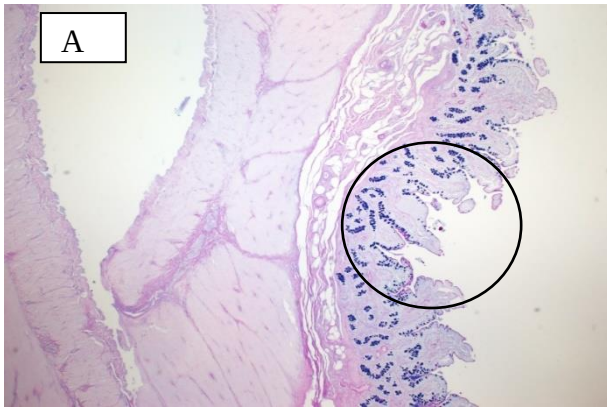
8. Wash in lukewarm tap water for 5-10 minutes.

9. Dehydrate in two changes each of 75% and 100% ethyl alcohol. Clear in three changes of xylene, 10 dips each; coverslip with compatible mounting medium.

Counting procedure of goblet cells occupation area in mucosa (%):

1. Put the slides under the microscope (Olympus BX53).

2. Randomly choose 5 fields above submucosa (see circle area in figures A and B) under × 10 magnitude and count the marked goblet cells area portion in all crypts within each field using CellSens software. The mucous production was calculated by goblet cells occupation area in mucosa (%).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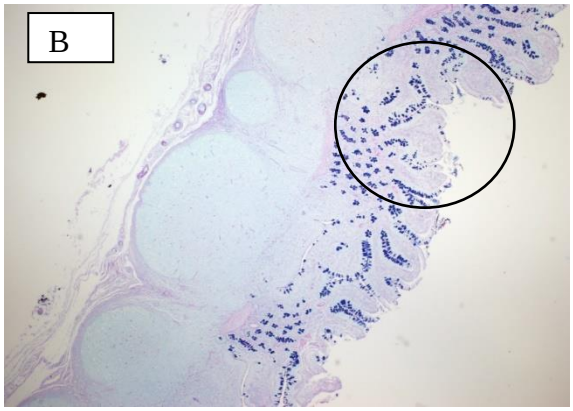
B

7.8 Purification of Total RNA Using the RNeasy Plus Universal Mini Kit (Qiagen)

1. Remove the tissue from -80ºC storage. Determine the amount of tissue. Do not use more than 50 mg tissue.

2. Disrupt the tissue and homogenize the lysate using the homogenizer (Kinematica, CH-6010 Kriens-Lu). Place the tissue in a suitably sized vessel containing 900 µL QIAzol lysis Reagent.

3. Place the tube containing the homogenate on the benchtop at room temperature (15-25 ºC) for 5 min.

4. Add 100 µL gDNA Eliminator Solution. Securely cap the tube containing the homogenate, and shake it vigorously for 15s.

5. Add 180 µL chloroform. Securely cap the tube containing the homogenate, and shake it vigorously for 15s.

6. Place the tube containing the homogenate on the benchtop at room temperature for 2-3 min.

7. Centrifuge at 12,000 × g for 15 min at 4 ºC. After centrifugation, heat the centrifuge to room temperature (15-25 ºC) if the same centrifuge will be used in the later steps of this procedure.

8. Transfer the upper, aqueous phase (usually 600 µL) to a new microcentrifuge tube.

9. Add 1 volume (usually 60 µL) of 70% ethanol, and mix thoroughly by pipetting up and down. Do not centrifuge. Proceed immediately to next step.

10. Transfer up to 700 µL of the sample to an RNeasy Mini spin column placed in a 2 mL collection tube (supplied). Close the lid gently, and centrifuge for 15 s at 10,000 rpm at

room temperature (15-25 ^{o}C). Discard the flow-through. Reuse the collection tube in next step.

11. Repeat step 10 using the reminder of the sample. Discard the flow-through. Reuse the collection tube in next step.

12. Add 700 µL Buffer RWT to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 10,000 rpm to wash the membrane. Discard the flow-through. Reuse the collection tube in next step.

13. Add 500 µL Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 10,000 g rpm to wash the membrane. Discard the flow-through. Reuse the collection tube in next step.

14. Add 500 µL Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at 10,000 g rpm to wash the membrane.

15. Place the RNeasy spin column in a new 2 mL collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.

16. Place the RNeasy spin column in a new 2.0 mL collection tube (supplied). Add 30-50 µL RNase-free water directly to the spin column membrane. Close the lid gently. To elute the RNA, centrifuge for 1 min at 10,000 rpm. Transfer the RNA to a new 1.5 mL collection tube (supplied).

17. Storage in -80^{o}C.

7.9 Using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems)

Prepare the 2 × RT master mix using the kit components before preparing the reaction plate:

1. Allow the kit components to thaw on ice.

2. Referring to the table below, calculate the volume of components needed to prepare the required number of reactions. Note: Prepare the RT master mix on ice.

Component	Volume/Reaction (µL)	
	Kit with RNase Inhibitor	Kit without RNase Inhibitor
10 × RT Buffer	2.0	2.0
25 × dNTP Mix (100 mM)	0.8	0.8
10 × RT Random Primers	2.0	2.0
MultiScribe™ Reverse Transcriptase	1.0	1.0
RNase Inhibitor	1.0	-
Nuclease-free H_2O	3.2	4.2
Total per Reaction	10.0	10.0

Preparing the cDNA reverse transcription (RT) reactions:

1. Pipette 10 µL of 2 × RT master mix into each well of a 96-well reaction plate or individual tube.

2. Pipette 10 µL of RNA sample into each well, pipetting up and down two times to mix.

3. Seal the plates or tubes.

4. Briefly centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles.

5. Place the plate or tubes on ice until you are ready to load the thermal cycler.

Performing reverse transcription:

1. Program the thermal cycler conditions, which are optimized for use with the High Capacity cDNA Reverse Transcription Kits.

	Step1	Step2	Step3	Step4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

2. Set the reaction volume to 20 µL.

3. Load the reactions into the thermal cycler.

4. Start the reverse transcription run.

7.10 Power SYBR Green PCR Master Mix: Performing Real-Time PCR Assays (Applied Biosystems)

Prepare the PCR master mix

1. Allow the Power SYBR® Green PCR Master Mix to thaw completely.

2. In a polypropylene tube, prepare the PCR master mix by scaling the volumes listed below to the desired number of PCR reactions. Note: include extra volume to account for pipetting losses.

Reaction component	Volume (µL)/Reaction (96-well Standard Plate)	Final Concentration
Power SYBR Green PCR Master Mix (2X)	10	1 X
Reverse primer	1	50 to 500 nM
Forward primer	1	50 to 500 nM
Template	2	100 ng
Nuclease-free water	6	-
Total volume	20	-

3. Mix gently. Do not vortex. Centrifuge briefly, and then prepare the PCR reaction plate.

Set up the plate parameters

Run the PCR reaction plate

1. Load the plate into PCR machine.

2. Open StepOne™ Software.

3. Advanced set up:

a). Instrument: StepOnePlus™ instrument (96 wells)

b). Type of experiment: Quantitative Comparative CT (ΔΔCT)

c). Reagents use to detect the target sequence: SYBR® Green Reagents

d). Ramp speed to use in the instrument run: Standard (about 2 hours to complete a run)

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