Feeding strategies for weaned pigs

by

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ABSTRACT

Weaning imposes a variety of stress factors on piglets including consuming dry feed instead of milk, changes of environment and physiology, and pathogenic challenges while decreasing passive immunity. Feeding strategies are cost-effectively attractive approaches to maintain health and promote growth in weaned pigs. While canola co-products are substitutes for soybean meal to reduce feed cost, fermented wheat holds significant potential to design a feeding strategy to promote growth and provide health benefits to weaned pigs. In Chapter 2, yellow-seeded *Brassica* (*B.*) *juncea* canola meal (CM) had greater fermentability and CATTD of gross energy than *B. napus* CM, but digestibility of amino acids did not differ, when fed to ileal-cannulated growing pigs. Fermentability of *B. napus* CM but not *B. juncea* CM decreased in the pig intestine with increased dietary inclusion up to 500 g/kg. Hence, yellow-seeded *B. juncea* CM had a greater DE value, similar amino acid digestibility as conventional black-seeded *B. napus* CM and may limit protein fermentation in the pig intestine. In Chapter 3, increasing inclusion of extruded *B. juncea* expeller at 0, 60, 120, 180 and 240 g/kg linearly decreased diet nutrient digestibility and energy values and quadratically increased overall ADFI and ADG, but did not affect overall G:F in weaned pigs. The linear increase of ADFI and ADG was curved at 240 g extruded *B. juncea* expeller/kg associated with increased dietary glucosinolates intake that prevented further increases in ADFI. In Chapter 4, six diets including 200 or 500 g/kg fermented wheat grain with *Lactobacillus reuteri* did not affect gut morphology, intestinal fermentation, growth performance, and CATTD of nutrients in weaned pigs. However, exopolysaccharides (EPS), reuteran or levan, demonstrated prebiotic effects by stimulating hindgut fermentation. Moreover, feeding fermented wheat with *L. reuteri*, especially with the presence of reuteran, reduced the copy numbers of genes for *E. coli* and its heat-stable enterotoxin in the intestinal

digesta (Chapter 6). In conclusion, *B. juncea* CM had greater energy value and fermentability than conventional CM. Feeding fermented wheat with *L. reuteri* that produce exopolysaccharides may promote gut health benefits to weaned pigs.

PREFACE

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DEDICATION

To my parents and my family for their unconditional love and support.

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List of Abbreviations

AA	amino acid(s)
ADFI	average daily feed intake
ADF	acid detergent fibre
ADG	average daily gain
AID	apparent ileal digestibility
ATTD	apparent total tract digestibility
B.	*Brassica*
BCFA	branched-chain fatty acids
BW	body weight
CAHF	coefficient of apparent hindgut fermentation
CAID	coefficient of apparent ileal digestibility
CATTD	coefficient of apparent total tract digestibility
cfu	colony-forming unit
CM	canola meal
CP	crude protein
CSID	coefficient of standardised ileal digestibility
d	day
DDGS	distiller's dried grains with solubles
DE	digestible energy
DM	dry matter
E. coli	*Escherichia coli*

ETEC	enterotoxigenic *Escherichia coli*
EPS	exopolysaccharides
FOS	fructo-oligosaccharides
GALT	gut-associated lymphoid tissue
GE	gross energy
G:F	feed efficiency (ADG/ADFI)
GOS	galacto-oligosaccharides
GPR43	G protein-coupled receptor 43
IL	interleukin
LPS	lipopolysaccharide
Lys	lysine
MOS	mannan-oligosaccharides
NDF	neutral detergent fibre
NE	net energy
NSP	non-starch polysaccharides
OF	oligofructose
PWD	post-weaning diarrhea
SID	standardised ileal digestible
SCFA	short-chain fatty acids
TNF-α	tumour necrosis factor-α
VFA	volatile fatty acids

CHAPTER 1 – Introduction

1.1. General introduction

Weaning imposes a variety of stress factors on piglets including consuming dry feed instead of milk, changes of environment and physiology, and pathogenic challenges while decreasing passive immunity. These sudden changes together with both an immature digestive tract and immune system can cause piglets to become susceptible to post-weaning diarrhea (PWD) (Lallès et al., 2007). Diarrhea leads to economic losses due to high morbidity and (or) mortality and impaired growth rate in affected pigs. In Canada (Ontario) and Finland, PWD outbreaks peaked during the first two weeks after weaning with 73 and 81% morbidity in piglets among studies (Amezcua et al., 2002; Laine et al., 2008). Mortality increased on infected farms from 2 to 7% after the PWD outbreak (Amezcua et al., 2002). Likewise, PWD occurred mostly within 2-4 weeks after weaning and the incidence was highest during the second week post weaning in 2005 to 2009 in Bulgaria, whereby morbidity ranged from 19 to 52% and mortality from 1.2 to 11.7% (Lyutskanov, 2011).

Weaned pigs have been fed diets with high density of nutrients to compensate for their low feed intake. However, to improve digestive and absorptive capacity of the gut, it is important to keep the gut filled with feed materials (Pluske et al., 1997). Inclusion of fibre components to the diet may exert positive effects on gut maturation, nutrient digestibility and growth performance of weaned pigs. In fact, addition of 40 g/kg wheat bran and 20 g/kg sugar beet pulp increased gut function and growth performance in weaned pigs (Hermes et al., 2009). With respect to the increased use of co-products in swine diets to reduce feed cost, it is essential to

understand the role of different types and levels of fibre in diets to optimize their benefits in swine feeding.

Feed antibiotics have been most commonly used in North America at the sub-therapeutic level to prevent PWD and gastrointestinal disorders. Other practices include vaccination, feeding strategies, dietary modifications, biosecurity and management measures (Fairbrother et al., 2005). However, antibiotics as growth promoters (AGP) were prohibited in animal feed across Europe since 2006 and extensively banned or restricted in other countries due to increasing concerns about human bacterial resistance to antibiotics (EU Commission press releases, 2005). Withdrawal of prophylactic antibiotics increased piglet mortality by approximately 30% as a consequence of increased rate of PWD in Denmark (Stein, 2002). Furthermore, growth performance was reduced and therapeutic use of antibiotics increased, because morbidity increased (Stein, 2002). To minimize effects of antibiotic removal, alternative practices are required to maintain health and performance of pigs (Thacker, 2013).

In this regard, gut health and feeding strategies attract increasing attention due to their essential roles during the course of diseases and growth of pigs. Gut health refers to the homeostasis and state of well-being of the gastrointestinal tract including normal and stable microbiota and effective immune status and functions (Pluske et al., 2007; Bischoff, 2011). A wide range of feed additives may enhance gut health such as probiotics, prebiotics, acidifiers, essential oils, and enzymes. Nonetheless, their effectiveness varies and single feed additives are not perfect replacement to antibiotics (Thacker, 2013). Thus, pig producers still require a feeding strategy that effectively exerts health benefits and cost-effectively promote growth in weaned pigs.

1.2. Canola co-products

Canola meal, a co-product of the canola oil industry, is globally ranked as the second most common supplemental protein feedstuff after soybean meal (Newkirk, 2009). CM contains 360-390 g/kg crude protein (as fed) with a well-balanced amino acid profile (Spragg and Mailer, 2007; Newkirk, 2009; Khajali and Slominski, 2012; NRC, 2012; Slominski et al., 2012). However, the lower protein content, greater proportion of fibre and presence of glucosinolates in CM make its nutritional value lower than that of soybean meal (Bell, 1993). CM has a relatively high fibre content because the outer seed coat (hull) is tightly adhered to the cotyledons, which does not separate during seed pressing to extract the oil (McCurdy and March, 1992). The hull contains approximately 60% dietary fibre and constitutes 30% of CM by weight (Bell, 1993; Slominski et al., 2012). Glucosinolates are major anti-nutritional factors in CM that may confer a bitter taste that may reduce feed intake and growth in pigs (Newkirk, 2009). Reduction in fibre and glucosinolates in canola meal is possible through plant breeding and processing (Bell, 1993; Mejicanos et al., 2016).

1.2.1. Canola species

In Canada, CM is mainly derived from *Brassica napus* (>95%), together with two other recognized species, *Brassica rapa* and *Brassica juncea* (Newkirk, 2009). As a crop, yellow-seeded *B. juncea* is more resistant to diseases and more tolerant to heat and drought stress; thus, better adapted to dry agronomic conditions than *B. napus* (Woods et al., 1991; Gan et al., 2007). Therefore, *B. juncea* is primarily targeted to grow in the Brown and Dark Brown soil zones of the North American Great Plains where conventional *B. napus* productivity is limited (Miller et al., 2003).

B. juncea has a thinner seed coat and thus contains less fibre than conventional black-seeded *B. napus* canola (Khajali and Slominski, 2012). *B. juncea* CM has a greater content of glucosinolates than *B. napus* CM, especially gluconapin that was associated with 10% decreased feed intake in young pigs when fed up to 240 g *B. juncea* CM/kg (Landero et al., 2013). *B. juncea* CM contains less lignin and polyphenols and more pectic polysaccharides (304 vs. 266 g/kg) than *B. napus* CM (Slominski et al., 2012). In addition, the slightly greater content of oligosaccharides (36 vs. 31 g/kg) in *B. juncea* CM than *B. napus* CM facilitates fermentation and thereby contributes more energy in the form of volatile fatty acids (VFA) (Jia et al., 2012). Furthermore, the greater CATTD of ADF (0.67 vs. 0.41) and NDF (0.76 vs. 0.52) of *B. juncea* CM than *B. napus* CM indicated that *B. juncea* CM was fermented better than *B. napus* CM (Zhou et al., 2015).

1.2.2. Canola meal processing

The nutritional value of CM is also affected by processing conditions. Oil is primarily pressed from canola seed by expeller or cold-press extraction (Newkirk, 2009). Solvent extraction is the most commonly used follow up processing step in large-scale crushing plants to produce canola oil for human consumption and the co-product CM. The process generally involves: 1) seed cleaning to remove foreign materials, 2) preconditioning to bring the seed to 30-40^{o}C that prevents seed fractures causing reduction in oil extraction, 3) flaking to rupture cell walls and release oil, 4) steam cooking to increase temperature of the flakes to 80-105^{o}C that facilitates lipid coalescing by reducing oil viscosity, 5) expeller pressing to extract 60-70% of the oil from canola flakes, 6) hexane solvent extraction to extract the remaining oil, 7) steam desolventizing to remove remaining solvents in the meal, and 8) toasting at 95-115^{o}C before drying and cooling

(Unger, 1990; Newkirk, 2009). Gums are added back to the meal. This is the most efficient oil extraction, leaving only 17-50 g/kg of ether extract in the meal (Newkirk, 2009; Landero et al., 2011, 2013).

Expeller or cold-pressing without solvent extraction is conducted in small-scale or on-farm crushing plants where canola oil is merely mechanically pressed out producing co-products canola expeller and canola press-cake, respectively. For expeller pressing, canola seed is subjected to cleaning, conditioning, flaking, cooking and expelling. The temperature generated by friction in the screw press is 100-135^{o}C among plants. Canola seed may also be extruded prior to expelling to increase the efficiency of oil extraction. The resulting canola expeller contains 80-170 g/kg remaining oil (Newkirk, 2009; Woyengo et al., 2010; Landero et al., 2012; Grageola et al., 2013; Le et al., 2014). In cold press extraction, canola seed are cleaned and subjected to screw pressing at lower temperature (<70^{o}C) without prior conditioning, flaking and cooking. The remaining oil content in canola cold-press cake is 100-240 g/kg (Newkirk, 2009; Seneviratne et al., 2011; Grageola et al., 2013; Zhou et al., 2016).

1.2.3. Chemical composition of canola meal

The chemical composition of *B. juncea* and *B. napus* CM compared with that of soybean meal is shown in Table 1. The acid detergent fibre (ADF) content is 120-190 g/kg; whereas neutral detergent fibre (NDF) content is 180-260 g/kg. The total dietary fibre of CM is 240-310 g/kg, including 18-20% non-starch polysaccharides (NSP), 3.7-10% lignin with associated polyphenols and 1.5-3.3% glycoprotein. CM contains more total dietary fibre, less soluble NSP and less oligosaccharides than soybean meal (Bell, 1993; Khajali and Slominski, 2012; Slominski et al., 2012; Woyengo et al., 2016).

1.2.4. Antinutritional factors

1.2.4.1. Glucosinolates

Glucosinolates are sulphur-containing secondary plant metabolites in *Brassicaceae* families (Tripathi and Mishra, 2007). More than 120 types of glucosinolates have been identified according to the variable amino acid side chain in the structure with a common group of β-D-thioglucose and a sulfonate oxime residue. Intact glucosinolates are relatively non-toxic (Bell, 1993). Glucosinolates and their hydrolytic enzyme, myrosinase, are present in canola seeds in separate cellular compartments. They come into contact to start the hydrolysis when the seed is mechanically pressed during crushing (Tripathi and Mishra, 2007). Glucosinolate degradation is also triggered by microbial activity in the hindgut, heat treatment or at low pH condition (Bell, 1993). Glucosinolates are hydrolysed to a wide range of breakdown products according to glucosinolate types and reaction conditions. They include isothiocyanates, goitrin, nitriles and thiocyanates (Tripathi and Mishra, 2007).

Type and level of glucosinolates and their degradation products exert differential effects. High dietary content of glucosinolates induces liver and thyroid enlargement, iodine deficiency, imbalance of serum thyroid hormones in pigs and reduction in growth performance (Schöne et al., 1997a; Tripathi and Mishra, 2007). The major glucosinolate in *B. juncea* is gluconapin and in *B. napus* is progoitrin (2-hydroxy-3-butenyl; Tripathi and Mishra, 2007; Slominski et al., 2012). Gluconapin tastes bitter with greater intensity than progoitrin (Fenwick et al., 1983). Gluconapin content was negatively correlated with growth performance and CP digestibility (Landero et al., 2013) in weaned pigs. Thus, the level of glucosinolates should not exceed 2.4-2.5 mmol/kg for pig diets (Bell, 1993; Schöne et al., 1997b).

1.2.4.2. Phytate, tannins, sinapine

Phytate, a mixed salt of phytic acid, is the main storage form of phosphorus in plant seed. Phytate is an anti-nutritional factor in animal feed due to its chelation of minerals and protein (Selle and Ravindran, 2008). CM contains 30-60 g/kg phytate (Bell, 1993). Although phosphorus content is greater in CM (11.3 vs. 7.3 g/kg) than in soybean meal, it is mostly present as phytate (Khajali and Slominski, 2012).

Tannins are complex phenolic compounds, including hydrolysable and condensed tannins. Insoluble condensed tannins are predominant in canola hull (19–62 g/kg oil-free hulls), with greater content in black than yellow hulls (Naczk et al., 2000; Slominski et al., 2012). Tannins form complexes with proteins and proteolytic enzymes in the digestive tract that negatively affects protein digestion in animals (Khajali and Slominski, 2012). However, feed efficiency was improved in weaned pigs fed tannin-rich diets (Biagi et al., 2010).

Sinapine, an ester of sinapic acid and choline, consists of 6-18 g/kg in CM (Bell, 1993). Sinapine is responsible for the "fishy eggs" produced by susceptible hens that are unable to convert sinapine or choline metabolites to odourless compounds (Khajali and Slominski, 2012). Despite bitter flavour, sinapine is less important than glucosinolates in the depression of palatability and feed intake in pigs (Bell, 1993).

Table 1.1. Chemical composition of soybean meal and canola meal from black *B. napus* and yellow *B. juncea* (g/kg, DM basis)

Component	*B. juncea*[1]	*B. napus*[1]	Soybean meal[2]
Crude protein	474	438	507
Ether extract	17	18	14
Ash	72	73	71
Carbohydrates			
Monosaccharides	3	2	7
Sucrose	92	88	70
Oligosaccharides	36	31	62
Starch	3	4	26
Total dietary fibre	258	301	242
NSP	200	202	217[3]
Cellulose	57	57	62[3]
Non-cellulosic NSP	143	145	155[3]
NSP component sugars (% of total)			
Rhamnose	1.2	1.2	1.9[3]
Fucose	0.8	1.0	-
Arabinose	24.1	22.9	16.6[3]
Xylose	7.5	9.1	12.1[3]
Mannose	1.5	2.6	8.3[3]
Galactose	7.7	7.9	26.1[3]
Glucose	27.6	29.6	4.5[3]
Uronic acids	30.4	26.6	30.6[3]
Lignin and polyphenols	39	71	2.8
Glycoproteins	17	21	1.5
Soluble NSP	22	18	63[3]
Insoluble NSP	178	184	155[3]
ADF	128[4]	186[4]	83
NDF	195[4]	269[4]	133
Glucosinolates, μmol/g	17.2	27.1	-

[1] Slominski et al., 2012; [2] Khajali and Slominski, 2012; [3] Bach Knudsen, 1997; [4] Woyengo et al., 2016

1.3. Prebiotics

1.3.1. Definition

The term “Prebiotics” has been widely used in recent years indicating dietary ingredients that are not digested by digestive enzymes and give potential benefits to the host through selectively supporting the growth of certain beneficial microbiota in the gut thus improving the host health (Lomax and Calder, 2009).

1.3.2. Origin, structure and characteristics of prebiotics

Based on this definition, prebiotics are ingredients that share three features: (a) unable to be digested by endogenous enzymes in the small intestine; (b) fermentable by certain intestinal microbes when reaching the large intestine; (c) able to exhibit selective effects on microbes that subsequently improve health benefits (Figueroa-Gonzalez et al., 2011).

Prebiotic oligosaccharides are natural carbohydrates in plants, yeasts and breast milk. Prebiotics can be extracted from plants or synthesized by hydrolysis of polysaccharides using microbial or enzymatic methods (Figueroa-Gonzalez et al., 2011). Prebiotic oligosaccharides are mainly short-chain carbohydrates containing 3 to 10 sugar molecules that escape enzymatic digestion (Swennen et al., 2006). Some prebiotic oligosaccharides are β2-1 fructans [inulin (IN) and fructo-oligosaccharides, (FOS)]; galacto-oligosaccharides (GOS), gluco-oligosaccharides, isomalto-oligosaccharides, lactulose, mannan-oligosaccharides (MOS), oat β-glucan, soya-oligosaccharides (mixture of raffinose and stachyose) and xylo-oligosaccharides (Lomax and Calder, 2009; Figueroa-Gonzalez et al., 2011). Of these prebiotic oligosaccharides, inulin-type fructans are best known for their effects on bifidobacteria. Among studies, beneficial microbiota like bifidobacteria and lactobacilli grow better on oligofructose (OF) and inulin than on glucose

(Gibson et al., 2005). Moreover, some carbohydrates have prebiotic effects such as resistant starch and non-starch polysaccharides (Williams et al., 2001).

1.3.3. Possible mechanisms of action

Prebiotics have indirect effects on the host defense through selective modification of the intestinal microbiota profile. By facilitating intestinal fermentation and increasing the number of beneficial microbes, these microbiota directly compete with pathogenic bacteria for nutrients and attachment sites on gut epithelial barriers. The presence of this type of microbiota also benefits the host by activation of the gut mucosal immune system up to a certain level to fight pathogens (Lomax and Calder, 2009).

Fermentation of prebiotic oligosaccharides in the hindgut produces short-chain fatty acids (SCFA) that subsequently lower the pH of the intestinal environment to impair the growth of pathogens and facilitates mucin release to prevent pathogens from binding to mucosa (Licht et al., 2012). The SCFA, in turn, play a role in the activation of immune cells in the gut-associated lymphoid tissue (GALT). Especially, butyrate provides energy for enterocytes and contributes to mucosal immune signaling (Lomax and Calder, 2009).

1.3.4. Potential benefits of prebiotics

1.3.4.1. Benefits to the host

Prebiotics are capable of directly modulating immune effects *in vitro* and *in vivo*. Some prebiotic fibers act as stimulants and modulators to dendritic cells in response to lipopolysaccharide (LPS). The β-glucans and some other prebiotics are able to bind to enterocytes via surface receptors

dectin-1. Other effects of prebiotics on the immune system are indirect through stimulation of microbiota growth and production of SCFA (Licht et al., 2012).

The SCFA lower luminal pH subsequently limits the number of pathogenic bacteria that prefer alkaline environment (Wong et al., 2006). In addition to pH reduction, SCFA are able to modulate intestinal immune functions via recruitment of leukocytes to inflammatory sites by inducing neutrophil chemotaxis (directional migration to chemoattractants) dependent on the G protein-coupled receptor (GPR43). The SCFA also affect the production of cytokines such as TNF-α, IL-2, IL-6 and IL10 by leukocytes (Vinolo et al., 2011).

Dietary manno-oligosaccharides (MOS) increased cell turnover in villi crypts in grower-finisher pigs from 21 to 100 kg of body weight (Ewing, 2008). The SCFA produced by gut microbiota play important roles in proliferation of colonic cells. First, SCFA, especially butyrate, serve as an energy source for epithelial cells. Second, butyrate affects cell proliferation by releasing growth factors or gastrointestinal peptides or modulating mucosal blood flow. Finally, SCFA influence gene expression related to cell proliferation (Blottiere et al., 2003).

Addition of 30 g/kg FOS in the diet of weaned pigs may increase the production of total SCFA resulting in a pH reduction by 7% in the caecum and proximal colon. However, dietary supplementation of 2.5 g/kg FOS increased only the concentration of butyrate and isobutyrate (as % of total SCFA) (Shim et al., 2005). Butyrate from microbial fermentation is used as a source of energy for growth of colonocytes during its transportation across the epithelium (Guilloteau et al., 2010).

Growth rate of weaned pigs was effectively enhanced by the inclusion of MOS; and it was further improved when antimicrobial agents such as copper sulfate were supplemented in the diet (Cromwell, 2001). This effect can be explained by increased absorption of nutrients due to

the increase in villous height in the small intestine of piglets fed oligofructose (Loo and Vancraeynest, 2008). Empty body weight gain and dietary digestible energy were greater in weaned pigs consuming FOS at 2.5 and 30 g/kg compared with controls. In pigs fed 2.5 g/kg FOS, villi in the proximal small intestine were 24% longer compared with controls (Shim et al., 2005).

1.3.4.2. Reduction of pathogen loads in the gastrointestinal tract

Feeding prebiotic oligo- and polysaccharides (glycans) increased the population of beneficial microbiota in the pig intestine, neutralized bacterial toxins and impaired binding ability of pathogens to attach to the intestinal wall (Koropatkin et al., 2012). To cause infections, pathogens need to attach to the intestinal membrane via receptors that can be either occupied or structurally mimicked by some oligosaccharides (Figure 1; Shoaf- Sweeney and Hutkins, 2008). Therefore, pathogenic infections are inhibited when pathogenic bacteria bind to oligosaccharides instead of epithelial cells. Pectins and pectic oligosaccharides were able to reduce the effect of Shiga like toxins from *E. coli* O157:H7 that may result from bacterial binding to these specific oligosaccharides. This direct interaction between prebiotic oligosaccharides and pathogens is capable of adherent inhibition that may be related to regulation of virulence genes of pathogens (Licht et al., 2012).

The MOS typically bind to pathogens that contain fimbriae (type 1) sensitive to mannose receptors on enterocytes, such as *Salmonella* spp. and *E. coli*. By binding to MOS instead of colonizing the gut lining, these bacteria are eventually washed out from the gut lumen. The MOS provided benefits by reducing incidence of diarrhea from 2.9 to 1.7 % and increasing growth

performance of weaned piglets fed MOS for 21 days when compared with feeding the zinc bacitracin control diet (Ewing, 2008).

Ammonia is a product from the degradation of endogenous urea by bacteria such as *E. coli* in the gut. This catabolite is harmful to enterocytes and negatively affects microbiota. Increasing inclusion of FOS in diets for weaned pigs did not increase concentration of ammonia in the caecum and feces. Increased microbial fermentation in the gut uses more protein for growth, then more microbial protein and less ammonia is produced (Shim et al., 2005). Also, anaerobic bacteria use ammonia as a nitrogen source when fermentable carbohydrates are provided in the diet. Therefore, the lumen concentration of ammonia is reduced (Gaskins, 2001).

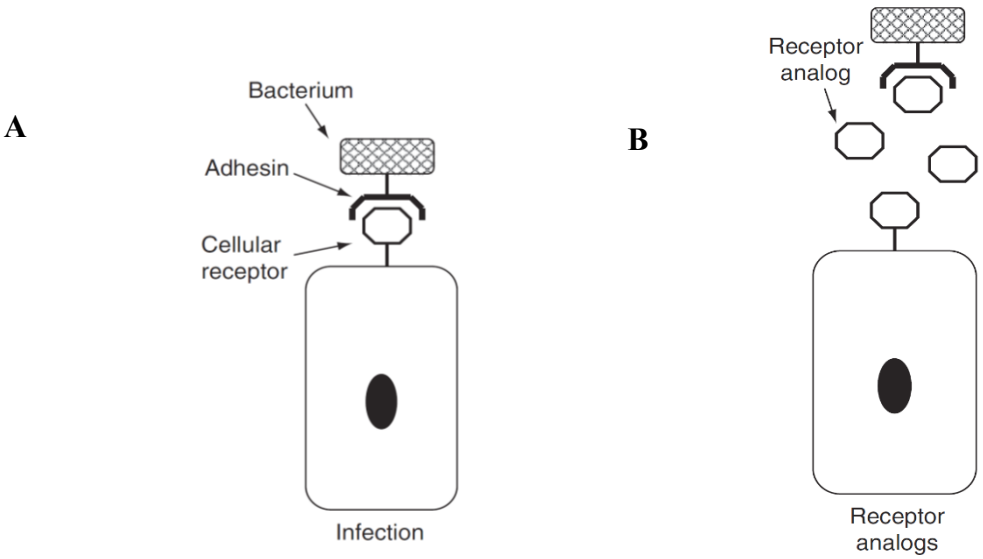

Figure 1.1. Proposed mechanism of competitive pathogen exclusion by oligosaccharides. Intestinal pathogens adhere to receptors on epithelial cells to trigger infection (A). Oligosaccharides act as receptor analogs that compete with pathogens for receptor binding sites (B) (Shoaf- Sweeney and Hutkins, 2008).

1.3.4.3. Influence on nutrient utilization

Nutritional effects of prebiotics can be obtained via metabolic functions of microbiota. The fermentation of indigestible components by microbiota provides the host a source of energy from undigested feed and from bacterial cells. The energy is then used by microbiota to expand their population. Main products of fermentation are SCFA also named volatile fatty acids (VFA), water and other compounds and gases such as hydrogen, carbon dioxide, and methane. The SCFA contribute up to 28% of the maintenance energy requirement of the pig. However, energy usage from SCFA is less efficient than energy derived from endogenous carbohydrate digestion (Wells et al., 2008; Garcia et al., 2010).

Besides, gut microbiota plays a role in synthesis of amino acids in the intestine and provide themselves as a source of protein for the host. Indeed, the first limiting amino acid, lysine is synthesised by microbiota and absorbed mostly in the small intestine of cannulated pigs where microbiota is present in smaller amount but with similar fermentation capacity to that in the large intestine (Torrallardona et al., 2003). Little attention has been paid to the quantity of amino acid production by microbes and how much microbial protein may contribute to porcine requirement of amino acids. Although the pig large intestine can absorb amino acids, the extent of absorption is influenced by the degree of microbial colonization (Gaskins, 2001).

The SCFA induce absorption of water, electrolytes and minerals in the gastrointestinal tract (Vinolo et al., 2011). The SCFA from fermentation of prebiotic carbohydrates such as resistant starch and inulin increased intestinal absorption and balance of Ca and Mg in rats, without affecting plasma levels of these minerals (Younes et al., 2001). B group vitamins such as vitamin B_{12}, biotin, thiamine and folate are synthesised in the hindgut by microbiota (Macfarlane et al., 2008).

An interesting effect of prebiotics is related to pork quality: pork sensory attributes. Boar taint is the accumulation of skatole (product of tryptophan fermentation) and androstenone in tissue. Feeding prebiotics such as inulin or OF to male pigs may reduce the production of skatole from tryptophan proteolytic fermentation in the gut (Loo and Vancraeynest, 2008).

1.3.4.4. Factors that influence prebiotic efficacy

The effects of prebiotics are different in individuals. It is because of several factors including type and dose of prebiotics, target bacteria species, the population of microbiota in individuals and different responses of individuals to prebiotics, duration of prebiotics usage, and interactions with other components in the diet (Lalles et al., 2007; Fanaro and Vigi, 2008; Loo and Vancraeynest, 2008). In addition, combinations of prebiotic carbohydrates may also have synergic effects (Younes et al., 2001).

1.3.5. Negative impacts of prebiotics in pigs

Despite many beneficial effects, prebiotics also bring undesirable effects to the host. During protection of the host from pathogen invasion, some prebiotics bind to specific receptors on the intestinal epithelial cells that irritate those cells. As a result, permeability of the intestinal mucosa is increased that subsequently causes the risk of bacterial translocation from the lumen (Licht et al., 2012). The high acidic environment in the gut due to excessive SCFA production of a large amount of microbiota also involves gut irritation.

Excessive SCFA in the lumen as a result of hyper-fermentation can lead to osmotic diarrhea. This effect was observed when weanling pigs were fed 30 g/kg of FOS instead of 2.5

g/kg (Shim et al., 2005). In addition, because prebiotics itself has osmotic effect in the gastrointestinal tract that may cause diarrhea in some cases (Marteau and Boutron-Ruault, 2002).

Other adverse effects come from catabolites of bacterial fermentation. Amino acid metabolites are toxic and affect the growth and differentiation of intestinal cells. As a result, pigs grow slower due to decreased nutrient absorption. Diarrhea in weaned pigs has been related to high amine production. Histamine concentration in the lumen also affects gut functions and consequently growth rate of pigs. As a vasoactive, histamine can cause contraction of smooth muscle and increase the secretion of gastric acid, mucosal blood flow, secretion of goblet cells and intestinal permeability (Gaskins, 2001).

Table 1.2. Effects of prebiotics on growth performance of weaned pigs

Experiments	Duration (day)	Feeding strategy	ADFI* (g)	ADG* (g)	Gain: Feed	Significant results	References
MOS* 0, 2, 3 g/kg	28	Dry 3-phase feeding	No	No	No	-	LeMieux et al., 2003
MOS* 0, 2 g/kg	35	Dry	No	No	↑	↓ Enterobacteria in jejunum	Castillo et al., 2008
FOS* 0, 2.5, 30 g/kg	21	Dry	No	No	No	↑ hindgut fermentation	Shim et al., 2005
GOS* FOS* (sugar beet pulp) FOS* (chicory inulin) At 10, 40 g/kg	15	Dry	-	-	-	↑ bifidobacteria in the caecum	Modesto et al., 2009
Inulin 50, 100, 150 g/kg Cellulose 50, 100, 150 g/kg	14	Dry	No	No	No	↓ pH, ↑ *Lactobacillus*: coliform in proximal colon	Wellock et al., 2007
Inulin 40 g/kg	35	Dry	No	No	No	↑ bifidobacteria, ↑ lactobacilli, ↓ Enterobacteria	Patterson et al., 2010
Inulin 40 g/kg	28	Dry	No	↑ (d22-28)	No	↑ total aerobes (stomach, jejunum) ↓ enterococci (colon)	Mair et al., 2010
Levan 0, 1 g/kg	28	Dry	No	↑	No	↑ nutrient digestibility (d0-14)	Zhang and Kim, 2014
β-glucan 10 g/kg	35	Dry 2-phase feeding	-	↑ (d1-14)	↑ (d1-14)	-	Lee et al., 2016

* ADFI: average daily feed intake; ADG: average daily gain**;** GOS: galactooligosaccharides; FOS: fructooligosaccharides; MOS: mannooligosaccharides

1.4. Probiotics

Probiotics was defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989). The most commonly used probiotics in animal feed include species of the genera *Lactobacillus, Bifidobacterium, Enterococcus, Bacillus* and *Saccharomyces*. These species share common characteristics including tolerance to low pH and bile acids, ability to grow in the gastrointestinal tract without causing side effects to the host, adhesion to intestinal mucosa, ability to inhibit growth of pathogenic bacteria by producing toxic substrates, and capacity of modulating innate immune responses (Lähteinen et al., 2010; Cho et al., 2011).

Potential health effects of probiotic bacteria result from possible actions: 1) improving intestinal barrier functions by inducing mucus secretion by goblet cells and modulating tight junction, 2) competitive exclusion of pathogens by competing with them for binding sites on intestinal mucosa and for nutrients in the intestinal lumen, and by producing short-chain fatty acids resulting in changing favourable environment for pathogens to survive, 3) inhibition of pathogenic replication by producing antimicrobial substrates such as bacteriocins, bacteriocyclin, and 4) modulation of immune responses (Brown, 2011).

Probiotics have been fed to weaned pigs to reduce the incidence of diarrhea and thus improve daily gain and feed efficiency in weaned pigs (Kyriakis et al., 1999). Together with improved feed efficiency, faecal enterobacteria and the number of eosinophils in intestinal tissues were found to decrease in weaned pigs given 3 mL of probiotic mixture of *L. amylovorus* and *E. faecium* (Ross et al., 2010). Furthermore, supplementation of a *L. reuteri* and *L. plantarum* complex at 1×10^9 CFU/kg for 4 weeks improved total tract digestibility of N and gross energy, increased weight gain and faecal *Lactobacillus*, reduced diarrhea, faecal *E. coli*,

and gas emission in weaned pigs (Zhao and Kim, 2015). Probiotics were also provided in liquid feed to prevent post-weaning diarrhea and improve growth performance in weaned pigs (Wang et al., 2012). However, efficacy of probiotics is influenced by several factors such as microbial strains, doses, combination with other probiotic strains, its stability in the feed, health conditions of animals, housing, duration and frequency of administration (Cho et al., 2011).

Table 1.3. Effects of probiotics on growth performance of weaned pigs

Experiments	Duration (day)	Feeding strategy	ADFI* (g)	ADG* (g)	Gain: feed	Significant results	References
L. plantarum 4.1 + *L. reuteri* 3S7, 10^{10} cfu/kg	15	Dry	-	-	-	↑ endogenous *Lactobacillus* ↓ enterobacteria	De Angelis et al., 2007
B. animalis subsp. lactis (Ra18) *B. choerinum* (Su891)	15	Dry	-	-	-	↑ caecal bifidobacteria ↑ caecal bifidobacteria	Modesto et al., 2009
Mixture 1×10^9 cfu/kg *E. faecium, L. salivarius, L. reuteri, B. thermophilum*	28	Dry	No	No	No	↑ enterococci in ileum, colon ↑ *Lactobacillus: enterococci* in colon	Mair et al., 2010
L. reuteri 10^{11} cfu/kg *B. subtilis* 10^{11} cfu/kg *L. reuteri* + *B. subtilis* 10^{11} cfu/kg	21	Dry	No No No	↑ ↑ No	↑ ↑ No	*L. reuteri* had no effect on diarrhea, *B. subtilis* ↓ diarrhea by 42%; no changes in IL-1β, TNF-α, IL-6; ↓ serum IgG, IgM	Wang et al., 2011
Feed base + fermented corn with - *L. acidophilus* 2×10^{10} cfu/kg or - *P. acidilactici* 10^{10} cfu/kg Natural fermented corn (control)	35	Wet (30-40% moisture)	No	↑ d15-35 (*L. acidophilus*)	No	↓ fecal coliforms in *L. acidophilus* (d14) than in *P. acidilactici* group, ↓ diversity, richness of colonic microbiota	Wang et al., 2012
L. reuteri + *L. plantarum* 1×10^9, 2×10^9 cfu/kg	28	Dry	No	↑	No	At 1×10^9 cfu/kg, ↑ CATTD* of N, GE, ↑ faecal *Lactobacillus*, ↓ gas emission, diarrhea score, *E. coli*	Zhao and Kim, 2015

* ADFI: average daily feed intake; ADG: average daily gain; cfu: colony-forming unit; CATTD: coefficient of apparent total tract digestibility; GE: gross energy.

1.5. Synbiotics

Synbiotics is the combination of probiotics and prebiotics where specific substrates are provided together with live microorganims (de Vrese and Schrezenmeir, 2008). This combination supports the growth of probiotic microorganisms in the gastrointestinal tract of the host with their available substrates for fermentation. Therefore, it may benefit the host more due to the synergistic effects of probiotics and prebiotics (Collins and Gibson, 1999). However, the efficacy of synbiotics is inconsistent due to changes in dietary composition, microbial composition in the gastrointestinal tract. Symbiotic applications are mostly reported for human.

Synbiotics are common combinations of probiotics with fructo-oligosaccharides, mannan-oligosaccharides, galacto-oligosaccharides, resistant starch, or other oligosaccharides such as maltodextrins, lactitol (Piva et al., 2005; Modesto et al., 2009; Mair et al., 2010; Chu et al., 2011). Feeding lactitol in conjunction with lactic acid bacteria, *Lactobacillus salivarius*, improved feed efficiency but did not affect average daily gain and feed intake in weaned pigs during 49-d trial after weaning (Piva et al., 2005). Although growth performance was not affected by supplementation of mannan-ooligosaccharides with one of *Aspergillus spp.*, *Saccharomyces spp.*, *Lactobacillus spp.*, digestibility of dry matter and crude protein was increased. In addition, faecal *E.coli* population and gas emission decreased (Chu et al., 2011). Regarding to combined effects of synbiotics, fermented feed may be the optimal alternative for weaned pigs.

Table 1.4. Effects of synbiotics on growth performance of weaned pigs

Experiments	Duration (day)	Feeding strategy	ADFI* (g)	ADG* (g)	Feed: gain	Significant results	References
No FOS* + no *B. longum* No FOS + *B. longum* FOS + no *B. longum* FOS + *B. longum*	19	Dry	No	↑[b]	No	↑ serum IGF-1* concentration[b]	Estrada et al., 2001
Control Lactitol 3 g/kg + *L. salivarius* 1B 4/11 10^8 cfu/kg	49	Dry	No	No	↑ (13%)[c]	-	Piva et al., 2005
FOS* (sugar beet bulp) at 0, 20 g/kg 2 strains: 0, 10^7, 10^9, 10^{11}cfu	15	Dry	-	-	-	↑ bifidobacteria in the caecum[b]	Modesto et al., 2009
B. animalis subsp. lactis (Ra18) *B. choerinum* (Su891)			No	No	No	↑ bifidobacterial/*E.coli* with increasing dose of *B. animalis subsp. lactis*[b]	
Control Inulin 4 g/kg Probiotics 10^9 cfu/kg (*E. faecium, L. salivarius, L. reuteri, B. thermophilum*) Inulin + probiotics	28	Dry	No	↑ (d22-28) [a, c]	No	↑ enterococci in the colon[b, c] ↑ lactobacilli: enterobacteria in the colon[c]	Mair et al., 2010
Control MOS* + *Aspargillus spp.* MOS + *Saccharomyces spp.* MOS + *Lactobacillus spp.*	15	Dry	 ↑ No ↓	 No No No	 No No No	↓ *E. coli* in feces[c], ↓ gas emission, ↑ digestibility of dry matter and crude protein	Chu et al., 2011

* ADFI: average daily feed intake; ADG: average daily gain; cfu: colony-forming unit; FOS: fructooligosaccharides; MOS: mannooligosaccharides; IGF-1: insulin-like growth factor-1

[a, b, c]: effects of prebiotics, probiotics, and synbiotics, respectively.

1.6. Organic acids in swine feeding

Organic acids have been widely used as food preservatives due to their ability to inhibit growth of bacteria and mold in feedstuffs (Freitag, 2007). Inclusion of organic acids and their salts in swine diets as potential alternatives to dietary antibiotics has gained more attention since antibiotics were banned as growth promoters (EU Commission press releases, 2005). Dietary supplementation of organic acids including formic, acetic, propionic, butyric, citric, fumaric, and lactic acid and their salts increased daily weight gain and feed efficiency in piglets during the first 3-4 weeks after weaning (Henry et al., 1985; Partanen and Mroz, 1999). Positive health effects of dietary supplementation of acids have been associated with reduced bacterial concentration in ileal digesta and suppression of post-weaning diarrhea in young pigs (Blank et al., 2001; Tsiloyiannis et al., 2001).

Organic acids are naturally present in cell metabolism (Freitag, 2007). Moreover, they are products of carbohydrate fermentation in the intestine of pigs. Dietary inclusion of organic acids is important to young pigs to reduce pH and coliform bacteria in the gastrointestinal tract, especially at weaning. Organic acids can act as antimicrobials by reducing pH below the growth range of pathogens. Undissociated forms of acid are able to cross bacterial cell membranes. Once in the cell, the acid dissociates at pH 7, then does suppress intracellular pH and decreases proton motive force causing cell death (Mroz, 2005; Niba et al., 2009a). The dissociation of acids in the gut help prevent pathogens from adhering to the brush border (Mroz, 2005). However, some bacteria such as *E. coli* are resistant to acids and able to survive at low pH.

However, efficacy of organic acids on health and growth performance of pigs is inconsistent due to the buffering capacity of diets, physicochemical forms of acid and delivery routes, fermentable carbohydrates and production of SCFA in the hindgut of pigs (Mroz, 2005).

Other factors such as corrosion of equipment, environmental impact and cost-effectiveness also affect the inclusion of organic acids in swine diets for a long period.

The antimicrobial activity of organic acids depends on the environmental pH, or the pK_a value, at which 50% of organic acids is dissociated. The buffering capacity increases from cereals to protein feedstuffs then mineral sources. Therefore, inclusion of diets with high buffering capacity suppresses the effect of organic acids on lowering pH, and as a consequence, reduces bactericidal effects (Partanen and Mroz, 1999). The form of organic acids and their salts, either liquid or solid, affects feed intake due to its palatability. Encapsulation of organic acids can help improve their taste, duration and sites of action (Mroz, 2005).

Many different feed additives exist to supplement diets for weaned pigs in place of antibiotics. However, responses to these additives are observed inconsistently under different raising conditions, levels of stress after weaning, composition of gastrointestinal microbiota and the growth of weaned pigs. It would be interesting to test the combination of additives mentioned in this review with a feeding strategy to promote growth performance in weaned pigs more efficiently and cost-effectively. In this regard, inclusion of fermented wheat with reuteran- or levan-producing *L. reuteri* in weaned pig diets may provide a combination of prebiotics reuteran or levan, probiotic *L. reuteri* and organic acids such as lactate and acetate.

1.7. Feed fermentation

Liquid feed is prepared by mixing dry ingredients or complete feed with water or liquid food industry by-products (Missotten et al., 2010a). As a result, the ratio of feed to water in liquid diets varies from 1:1.1 to 1:4 (Chae, 2000; Brooks, 2003). Liquid feed can be delivered to pigs

either immediately after mixing as non-fermented liquid feed (NFLF) or after a period of fermentation as fermented liquid feed (FLF).

Fermentation is the traditional method to preserve food by inhibition of spoilage organisms (Missotten et al., 2010a). Fermentation can be triggered by naturally occurring micro-organisms, such as yeast and lactic acid bacteria, in the mixture with water, creating spontaneous fermented liquid feed (SFLF). When fermentation is induced by a culture of selected lactic acid bacteria (LAB), it is referred to as inoculated fermented liquid feed (IFLF) (Plumed-Ferrer and Von Wright, 2009; Missotten et al., 2010a).

1.7.1. Properties of fermented feed

Fermented feed is characterized by low pH (below 4.5) due to increased concentration of lactic acid (above 150 mmol/l) and VFA, and high number of lactic acid bacteria (above 9 $\log_{10}$) (Niba et al., 2009a; Missotten et al., 2010a). In addition, the concentration of acetic acid and ethanol should be below 40 and 0.8 mmol/l, respectively.

1.7.2. Types of feed fermentation

1.7.2.1. Spontaneous fermentation

Spontaneous fermentation is induced by soaking feed ingredients in water for a period of time (Plumed-Ferrer and Von Wright, 2009). The fermentation initiates with low numbers of endogenous LAB, high pH, low concentration of organic acids and high population of yeast and coliforms. Later, fermentation reaches the steady state with high numbers of LAB, high concentration of organic acids, low pH and low numbers of coliforms. However, quality of

fermented products is inconsistent due to variation in the composition of endogenous microbiota in the feed ingredients, temperature, and duration of the fermentation process (Brooks, 2008).

1.7.2.2. Inoculated fermentation (controlled fermentation)

On the other hand, inoculated fermentation starts with an inoculum of concentrated LAB at the controlled temperature for a period of time (Plumed-Ferrer and Von Wright, 2009). In this case, fermentation is dominated by LAB, resulting in low pH, high concentration of organic acids, reduced coliforms, and controlled population of yeast.

1.7.2.3. Backslopping fermentation

Backslopping is the process where fermentation is inoculated by a portion of a previous fermentation (Brooks, 2008). As a result, the process of fermentation is accelerated and enables selection of the LAB population over time. However, there are risks of contamination by yeasts, which can reduce the nutritional value of fermented feed (Missotten et al., 2010a).

1.7.3. Fermentation of cereals

Fermentation of carbohydrate-rich ingredients, such as cereals, is preferable over fermentation of complete feed because of several advantages (Missotten et al., 2010a). First, fermentation of cereals is more controlled than that of complete feed due to their consistent composition and low buffering capacity resulting in rapid pH reduction and subsequent pH stabilization. Second, fermentation improves nutrient availability by activation of a variety of intrinsic cereal enzymes, leading to degradation of fibre and antinutritional factors in cereals (e.g. decrease in phytate resulting in increase in P availability) while it prevents the loss of synthetic amino acids and production of protein breakdown in complete feed (Missotten et al., 2010a). Fermentation of wheat by lactobacilli metabolises glutamine, glutamate, and arginine but not lysine (Gänzle,

2014). Finally, fermentation of cereal portion requires simple system in pig farms. However, fermentation of cereal portion needs to be inoculated with a LAB strain to obtain more lactic acid (Brooks, 2008).

1.7.4. Lactic acid bacteria

Lactic acid-producing bacteria (LAB) are a group of non-sporing, gram-positive bacteria that produce lactic acid as the major end-product from carbohydrate fermentation (König and Fröhlich, 2009). The LAB are divided into 2 groups based on their fermentation type. First, heterofermentative LAB apart from lactic acid also produce ethanol or acetate and CO_2. Second, homofermentative species only produce lactic acid as the main end-product (O'Bryan et al., 2014). The *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Tetragenococcus*, *Vagococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Oenococcus*, *Weissella* and *Streptococcus* are among 38 genera belonging to 6 families of LAB (Vandamme et al., 2014). The LAB are naturally-indigenous microorganisms detected in plant materials, in the gastrointestinal tract of humans and animals as commensal bacteria and in the environment (König and Fröhlich, 2009). However, LAB also include opportunistic pathogenic organisms among *Streptococcus* species (Holzapfel and Wood, 2014).

The LAB are commonly used in food fermentation as starter cultures to accelerate fermentation process and maintain quality of fermented food (Leroy and De Vuyst, 2004). Among LAB genera, *Lactobacillus* species are predominantly detected in fermented wheat sourdough, including obligate and facultative heterofermentative and obligate homofermentative species. The metabolic activities of these LAB determine properties of sourdough (Corsetti and Settanni, 2007).

One important feature of some *Lactobacillus* strains that recently gained more attention is the ability to produce exopolysaccharides (EPS). The EPS, including homopolysaccharides (HoPS) and heteropolysaccharides (HePS), improve sourdough texture and especially exert prebiotic effects (Corsetti and Settanni, 2007; Bounaix et al., 2009; Galle and Arendt, 2014). The HePS are formed intracellularly by polymerization of repeating units of 3 to 8 monosaccharides. On the other hand, the HoPS are extracellularly synthesized by converting sucrose into glucans and fructans in the presence of glucansucrases and fructansucrases, respectively. The HePS are mostly applied to improve the texture of fermented dairy products (Bounaix et al., 2009; Galle and Arendt, 2014). The LAB strains with ability to produce EPS are only detected in wheat and rye fermentation. The *L. reuteri* is one of four strains of wheat sourdough lactobacilli that produces reuteran (glucan) and levan (fructan) from sucrose (Tieking and Gänzle, 2005).

1.7.4.1. *Lactobacillus reuteri*

Lactobacillus reuteri is a symbiont of pigs that forms stable populations in the pars oesophagus. *L. reuteri* also occurs in cereal fermentations (Walter, 2008; Su et al., 2012). Most strains of *L. reuteri* produce EPS from sucrose. The EPS formation supports biofilm, structured microbial communities attached to cavity lining, formation by *L. reuteri* and is necessary for colonization of the upper intestinal tract of animals (Tieking and Gänzle, 2005). In the presence of sucrose as a substrate, *L. reuteri* TMW1.656 and LTH5794 produce the EPS reuteran and levan, respectively (Gänzle et al., 2007). Levan (*in vitro*) and reuteran (both *in vitro* and *in vivo*) inhibit adhesion of enterotoxigenic *Escherichia coli* (ETEC) K88 to porcine intestinal mucosa. Levan and reuteran also decrease net fluid loss induced by ETEC, indicating their potential to reduce the effect of post-weaning diarrhea induced by ETEC (Wang et al., 2010; Chen et al., 2014). In

addition, these bacteria also produce reutericyclin, an antimicrobial compound that inhibits bacterial growth (Gänzle and Vogel, 2003). *L. reuteri* possesses special properties to serve as probiotics. *L. reuteri* was found best adhesive to enterocytes among *Lactobacillus* isolates from pig intestine and feces (Lähteinen et al., 2010).

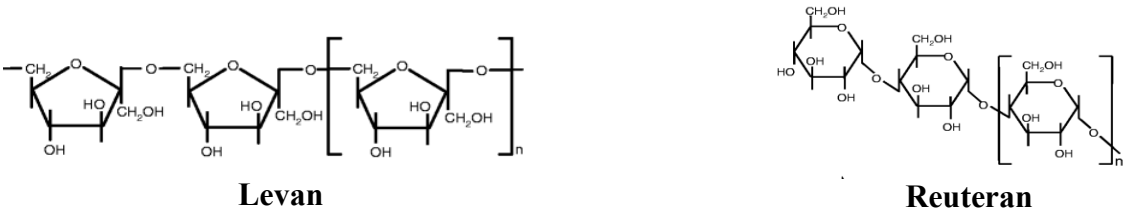

Figure 1.2. Structure of levan and reuteran (Korakli and Vogel, 2006).

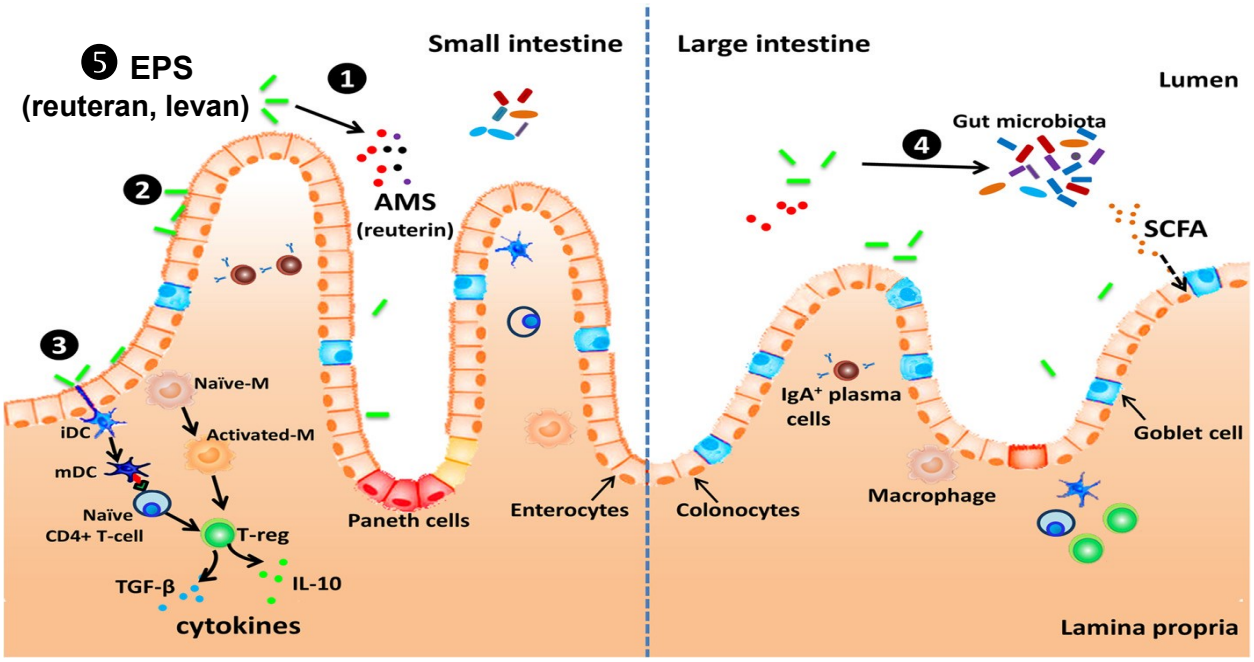

Figure 1.3. Mechanisms of modulating gut microbiota by *L. reuteri* in fermented wheat (adapted from Hou et al., 2015). 1) Secrete antimicrobial substances (AMS, such as lactic acid, reuterin, reutericyclin) 2) Colonize intestinal epithelial cells 3) Stimulate or suppress innate immune responses by cytokine production in macrophages (M), monocytes, and dendritic cells (DCs) 4) Produce short chain fatty acid (SCFA) 5) Produce exopolysaccharides (EPS) reuteran or levan.

1.7.4.2. Chemical changes in fermented wheat with *Lactobacillus reuteri*

During fermentation, the metabolic activities of heterofermentative LAB, such as *L. reuteri*, contribute to the activity of cereal enzymes to degrade carbohydrates, proteins and phenolic compounds in wheat grain. Subsequently, these breakdown products serve as substrates for sourdough bacterial growth. Although the low pH during fermentation inhibits maltogenic amylases, it promotes the activity of glucoamylases and xylanases in wheat to degrade starch and arabinoxylan, respectively (Gänzle, 2014). It was noted that fermentation of wheat with *L. sanfranciscensis* facilitated degradation of fructan and starch fractions by cereal enzymes (Korakli et al., 2002).

The increase in acidity during fermentation favors activities of intrinsic aspartic proteinases and carboxypeptidases that are mainly responsible for proteolysis in wheat doughs, resulting in the release of peptides and amino acids. The concentration of amino acids in sourdough is affected by microbial composition. While LAB preferably grow on peptides due to their own intracellular peptidases, yeast use amino acids for their growth (Gänzle et al., 2008). Some strains of LAB such as *L. reuteri* enable the conversion of arginine into ornithine via the arginine-deiminase pathway (ADI). Arginine and glutamine/glutamate conversions contributes to acid resistance of *L. reuteri* (Gänzle, 2015).

Moreover, the activation of cereal enzymes helps hydrolyze phytate in wheat dough (Gänzle, 2014). Phytate, a mixed salt of phytic acid and minerals, is the main storage form of phosphorus in plant feedstuffs. Phytate binds to divalent and trivalent cations, including calcium, magnesium and iron, to form insoluble complexes that escape digestive enzymes, especially at alkaline pH. Additionally, the phytate-protein complex may decrease digestibility of protein and amino acids (Gifford and Clydesdale, 1990; Honig and Wolf, 1991). However, phytate can be

degraded by phytase, which originates from the small intestinal mucosa, bacteria in the large intestine, cereal enzymes and inclusion of exogenous feed enzymes (Selle and Ravindran, 2008). About 95 to 100% of phytate is degraded in fermented wheat, which was associated with the activation of intrinsic phytase in wheat grains when pH was reduced as a result of lactic acid fermentation for 24 h at 30°C. The level of phytate hydrolysis was optimal around pH 5.5 and depended on the activity of cereal phytase (Reale et al., 2007).

1.7.5. Digestibility of nutrients in fermented feed

Wheat fermentation with *L. reuteri* provides several features which are similar to feed digestion in the stomach of pigs with the presence of lactic acid, organic acids, enzymes and commensal bacteria *L. reuteri*. Feeding fermented wheat contributes to digestion by maintaining the low gastric pH, which is important to weaned pigs for the protection against coliforms and initiating protein digestion, especially when secretion of HCl in the stomach is limited after weaning (Brooks et al., 2001; Yen, 2001).

The effects of fermented feed on growth performance of weaned pigs have been inconsistent. Whereas some studies showed improved performance in pigs fed FLF (Russell et al., 1996; Scholten et al., 2002), others found no effect (Geary et al., 1999; Canibe and Jensen, 2007; Cho et al., 2013). Ileal digestibility of dry matter, organic matter and energy of barley and wheat grain increased by 6 and 3%, respectively, in growing pigs fed fermented diets consisting of 800 g/kg fermented liquid barley or wheat grain and 200 g/kg of protein mixture (Jørgensen et al., 2010).

1.7.6. Effects of feeding fermented feed on growth performance

The effect of feeding fermented feed on growth performance of pigs is inconsistent. The effect on growth performance was not remarkable in weaned pigs fed fermented liquid feed with *Pediococcus acidilactici* (Geary et al., 1999). Pigs fed fermented liquid feed had lower feed intake and gained less than those fed non-fermented liquid feed (Canibe and Jensen, 2003). Palatability can be influenced by pH, concentration of organic acids produced during fermentation as well as the flavor and texture of the feed. High levels of acetic and butyric acid and biogenic amines resulted from spontaneous fermentation that reduced the palatability of pig feed (Beal et al., 2005; Canibe et al., 2010; Missotten et al., 2010a). Acidity influences the metabolism of LAB during fermentation, thereby affecting the viscoelastic properties of sourdough and taste of fermented feed through protein degradation (Gänzle et al., 2008; Gänzle, 2015).

Production of reuteran and levan by *L. reuteri* TMW1.656 and LTH5794, respectively, results from sucrose conversion that simultaneously increases the concentrations of acetate, mannitol and glucose in fermented wheat. In other words, the concentration of acetate is greater in wheat sourdough with added sucrose than that of sourdough without sucrose addition (Korakli et al., 2001; Galle et al., 2012). In Chapter 4 study, the acetate concentration was 51 ± 4 and 57 ± 4 mmol/kg of feed in wheat fermented by *L. reuteri* TMW1.656 and LTH5794, respectively. Thus, the sweet taste of fermented wheat consisting of reuteran or levan is reduced and the feed becomes less palatable to young pigs. It explains decrease in feed intake of pigs fed fermented diets with either reuteran or levan compared with those fed fermented diets without EPS.

1.7.7. Effects of wheat fermentation on gut microbiota and fermentation in the gut

1.7.7.1. Gut microbiota

One of the most important benefits of feeding fermented liquid feed to pigs is to reduce the incidence of potential enteropathogens such as *Salmonella* spp. and *Escherichia coli* (Missotten et al., 2010a). The biostatic and/or bactericidal effects of FLF are attributed to undissociated form of fermentation acids including lactic acid and VFA, fermentation metabolites such as bacteriocins, and competition between LAB and pathogenic organisms for food and binding sites along the gastrointestinal tract (Scholten et al., 1999).

The fermentation process eliminates spoilage organisms and pathogens present in ingredients or in the complete feed (Missotten et al., 2010a). When ingested, FLF reduces gastric pH which helps prevent the proliferation of pathogens invading the gastrointestinal tract (Brooks, 2008). The antimicrobial activity of fermented feed depends on the concentration of organic acids and metabolites produced during fermentation.

1.7.7.2. Intestinal fermentation

Fermentation of non-digestible oligosaccharides takes place in the large intestine where bacterial diversity is higher than that in the ileum (Montagne et al., 2003; Niba et al., 2009b). Levan, β-2,6 fructan, can be hydrolyzed under acidic conditions in the stomach to low molecular weight levan that then reaches the large intestines without further hydrolysis by digestive enzymes (Yamamoto et al., 1999). Low molecular weight levan, for instance, β-(2,6)-fructo-oligosaccharides (FOS), is more suitable for fermentation by bifidobacteria (Marx et al., 2000). Metabolism of β-(2,6)-FOS or fructose by bifidobacteria produces SCFA, mostly acetate (Marx et al., 2000).

Arabinoxylans are non-starch polysaccharides that are degraded by cereal enzymes to small chains with greater solubility during sourdough fermentation (Korakli et al., 2001). Soluble arabinoxylans are then fermented primarily in the caecum resulting in greater concentration of acetate and propionate than butyrate (Monsma et al., 2000; Belobrajdic et al., 2012). Fermentation of arabinoxylan in the hindgut exerts beneficial effects to the host by decreasing protein fermentation products. However, the extent of fermentation is influenced by the composition of arabionoxylan (Pollet et al., 2012).

1.7.7.3. Effects of fermentation on gut architecture

Villus height and villus height to crypt depth ratio are among standard indicators for gut health. The presence of feed in the intestinal lumen of pigs may stimulate cell proliferation, differentiation and turnover which then subsequently may affect the structure and functions of the intestinal epithelial layer. Hence, reduced feed intake at weaning is one of the major cause for villus atrophy in weaned pigs, not only because of the absence of feed in the gut but also energy deprivation (Pluske et al., 1997). It is supported by the fact that pigs fed FLF had lower dry matter intake, shorter villi and lower ratio of villus height to crypt depth than those on dry feed (Missotten et al., 2010b).

However, inclusion of fermented wheat in liquid diets to pigs enhances villus height and villus height to crypt depth ratio at the proximal part of the small intestine during the first week after weaning at 28 days of age (Scholten et al., 2002). The greater increase in villus height and crypt depth in the proximal than in the distal small intestine may be related to the amount of available nutrients in digesta in the lumen that distally decreases during transit (Vente-Spreeuwenberg et al., 2003).

Beneficial effects of fermented wheat in liquid diets over dry feed were observed by less reduction in villus height at day 4 and better recovery at day 8 after weaning (Scholten et al., 2002). In addition, activities of digestive enzymes also increased in response of fermented soybean meal, including protease and trypsin at the duodenum and jejunum of weaned pigs. Pigs fed FLF had lower gastric pH but higher small intestinal pH than those fed dry feed or liquid feed (Canibe and Jensen, 2003). It was more likely that low gastric pH induced secretion of pancreatic enzymes, resulting in increased pH in the small intestine.

1.7.8. Considerations of feeding fermented feed

Essential nutrients such as amino acids can be lost during fermentation due to the formation of biogenic amines. Cadaverine is formed from L-lysine, which is an irreversible process of decarboxylation. Fermentation of cereal before mixing complete feed prevents loss of essential nutrients. In addition, gastric ulcers were observed in pigs fed fermented feed (Missotten et al., 2010a).

1.7.9. Summary

Stress at weaning together with an immature digestive tract and immune system can cause piglets to become more susceptible to post-weaning diarrhea. Various feed additives have been studied for enhancement of gut health, but there are variations in their effectiveness and no single feed additive is capable of being a perfect replacement of antibiotics. Feed additives such as prebiotics, probiotics, and organic acids exhibit positive effects on health and growth performance of weaned pigs through their actions on fermentation and bacteria in the gastrointestinal tract of pigs. Variations in the effects of individual prebiotics, probiotics and

organic acids or effects of their combination on growth performance and gut health were associated with dietary composition, gut microbiota and interactions between them. Thus, it is necessary to include multiple additives at the appropriate proportions to maximize their synergetic effects on gut health and performance of weaned pigs.

Feed fermentation has become more common in swine production because of increasing demand for antibiotic alternatives. Fermentation of cereals is preferable due to its advantages in rapid fermentation process, maintaining nutrients, degrading non-starch polysaccharides, and producing prebiotic oligosaccharides and antimicrobial substrates. The start culture is required to remain quality of fermented feed. *L. reuteri*, a symbiont of pigs, also occurs in cereal fermentations. *L. reuteri* produce exopolysaccharides from sucrose. *L. reuteri* TMW1.656 and LTH5794 produce the EPS reuteran and levan, respectively. Levan and reuteran inhibit adhesion of ETEC K88 to porcine intestinal mucosa and decrease net fluid loss induced by ETEC. In addition, *L. reuteri* TMW1.656 also produce reutericyclin, an antimicrobial compound that inhibits bacterial growth. Therefore, fermented wheat with these strains of *L. reuteri* appears to offer promising improvement in young pig performance.

1.8. Gaps in knowledge

Dietary fermentable fibre may contribute up to 25% of energy requirements in the form of short-chain fatty acids and ameliorate negative effects of protein fermentation in the gut (Yen et al., 1991; Awati et al., 2006). Thus, better understanding of energy digestibility and hindgut fermentation of two main species of CM, *B. napus* and *B. juncea*, is essential. Canola expeller contains more ether extract than CM (Beltranena and Zijlstra, 2011) and may be valuable for weaned pig feeding. However, its feeding has not been studied. Feeding fermented wheat with

reuteran- or levan-producing *L. reuteri* showed effects on modulation of gut bacteria as described in previous studies (Yang et al., 2015a; Yang et al., 2015b). Nevertheless, there is little information on its effects on weaned pig growth performance, nutrient digestibility and characteristics of gastrointestinal morphology and fermentation.

1.9. Research hypotheses and objectives

1.9.1. Hypotheses

The hypotheses of the thesis were:

a) Feeding either *B. juncea* or *B. napus* CM to ileal-cannulated growing pigs at two inclusion levels (250 or 500 g/kg) would not affect energy and AA digestibility and intestinal fermentability.
b) Feeding weaned pigs with increasing inclusion of extruded *B. juncea* expeller formulated to equal NE and standardised ileal digestibility (SID) amino acid content would not affect nutrient digestibility and growth performance.
c) Feeding fermented wheat grain with *L. reuteri* TMW1.656 or LTH5794 with and without EPS to weaned pigs would not affect gut morphology, intestinal fermentation, nutrient digestibility, and growth performance.

1.9.2. Objectives

The overall objectives of this research were to investigate the effects of nutritional approaches to weaned pigs including diet modification and feeding fermented wheat.

a) To review the use of CM, fermented feed and their effects on the growth performance, gut health of weaned pigs (Chapter 1).

b) To investigate the digestibility of CM from 2 canola species, *B. napus* and *B. juncea*, in cannulated growing pigs (Chapter 2).

c) To investigate the effect of feeding extruded *B. juncea* canola expeller on growth performance and nutrient digestibility in weaned pigs (Chapter 3).

d) To investigate the effect of feeding fermented wheat with *L. reuteri* on growth performance, nutrient digestibility, gut morphology and fermentation products in weaned pigs (Chapter 4).

1.10. References

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CHAPTER 2 – Digestibility and intestinal fermentability of canola meal from *Brassica juncea* and *Brassica napus* fed to ileal-cannulated grower pigs

2.1. Abstract

Yellow-seeded *Brassica* (*B.*) *juncea* is a novel canola species. Therefore, its meal co-product requires feed quality evaluation and comparison to conventional, dark-seeded *B. napus* canola meal for pigs. The *B. juncea* canola meal contains less fibre than *B. napus* canola meal (190 vs. 260 g NDF/kg, as is), but also less lysine (20.3 vs. 22.1 g/kg). Nutrient digestibility and fermentibility of *B. juncea* and *B. napus* canola meal were assessed in a 2 × 2 factorial arrangement. Six ileal-cannulated pigs (47 kg BW) were fed six diets in a 6 × 6 Latin square: basal diet (460 g wheat/kg and corn starch), 4 diets with 460 g wheat/kg and either *B. juncea* or *B. napus* canola meal at 250 or 500 g/kg replacing corn starch, sugar and canola oil, and an N-free diet based on corn starch. The *B. juncea* canola meal had greater ($P < 0.05$) CATTD of gross energy than *B. napus* canola meal (0.70 vs. 0.63) most likely due to its lower fibre content. Ileal total VFA concentration was lower ($P < 0.001$) in pigs fed *B. juncea* than *B. napus* canola meal diets (15.2 vs. 20.8 μmol/g of wet digesta). In pigs fed *B. juncea* canola meal instead of *B. napus* canola meal diets, the molar ratio was greater ($P < 0.01$) for digesta propionate and faecal acetate, but lower ($P < 0.05$) for digesta and faecal butyrate. Canola meal species did not affect the CAID of gross energy, CSID of amino acid and faecal VFA content. The digestible energy (DE) value was greater ($P < 0.01$; 12.1 vs. 10.9 MJ/kg, standardised to 100 g/kg moisture) for *B. juncea* than *B. napus* canola meal. Increasing dietary inclusion of canola meal up to 500 g/kg reduced ($P < 0.01$) diet digestibility of gross energy but not amino acids and decreased ($P < 0.05$) intestinal fermentability of *B. napus* but not *B. juncea*. In conclusion, *B. juncea* canola

meal had greater fermentability and ATTD of gross energy than *B. napus* canola meal, but digestibility of amino acids did not differ. Increasing dietary inclusion of canola meal up to 500 g/kg reduced digestibility of gross energy but not digestibility of AA. Fermentability of *B. napus* canola meal but not *B. juncea* canola meal decreased in the pig intestine with increased dietary inclusion. Hence, yellow-seeded *B. juncea* canola meal had a greater DE value, similar amino acid digestibility as conventional dark-seed *B. napus* canola meal and may limit protein fermentation in the pig intestine.

Keywords*:* Canola meal; Digestibility; Energy; Pig; Volatile fatty acid

2.2. Introduction

Dietary inclusion of alternative feedstuffs such as co-products in swine diets has increased to reduce feed cost (Woyengo et al., 2014). As co-product of the food oil industry, canola meal can substitute soybean meal but has a lower energy value and contains less digestible amino acids (Newkirk, 2009). The difference with soybean meal is partly due to the greater fibre content in canola meal that reduces digestibility of energy and amino acids (Bell, 1993). Energy digestibility of canola meal could be increased by reducing its fibre content or by increasing microbial fermentation of undigested residue in the hindgut.

Yellow-seeded *Brassica* (*B.*) *juncea* has a thinner seed coat and contains less fibre than conventional black-seeded *B. napus* canola (Khajali and Slominski, 2012). *B. juncea* is targeted to grow where thermotolerance, disease resistance and adaptation to dry agronomic conditions are required (Woods et al., 1991). *B. juncea* canola meal contains more glucosinolates than *B. napus* canola meal, especially gluconapin that was associated with 10% decreased feed intake in

young pigs fed up to 240 g *B. juncea* canola meal/kg (Landero et al., 2013; Le et al., 2014). However, growing pigs can be fed up to 500 g canola meal/kg in studies on nutrient digestibility and intestinal fermentation of fibre (de Vries et al., 2016). Little information is available on nutrient digestibility and intestinal fermentation of *B. juncea* and *B. napus* canola meal at normal and high inclusion levels in diets of growing pigs.

Dietary fermentable carbohydrates produce volatile fatty acids (VFA) that may constitute 67-74% of energy absorbed in the hindgut of pigs (Anguita et al., 2006) and may contribute up to 25% of energy required (Yen et al., 1991). Dietary fermentable fibre may ameliorate negative effects of protein fermentation in the gut by using harmful nitrogenous metabolites, e.g., ammonia (NH_3), as N source (Awati et al., 2006). Better understanding of digestibility of nutrients of these canola meals and VFA produced including from protein fermentation at increasing dietary inclusion and subsequent fermentation is required to optimise energy use.

The objective of this study was to test the hypothesis that feeding either *B. juncea* or *B. napus* canola meal to ileal-cannulated growing pigs at two inclusion levels (250 or 500 g/kg) would not affect energy and AA digestibility and intestinal fermentability.

2.3. Materials and methods

Animal use and procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock and followed principles established by the Canadian Council on Animal Care (CCAC, 2009). The study was carried out at the Swine Research and Technology Centre, University of Alberta (Edmonton, AB, Canada).

2.3.1. Experimental animals

Six crossbred barrows (Duroc × Large White/Landrace F_1; Hypor, Regina, SK, Canada; 46.5 ± 0.84 kg initial BW) were surgically cannulated with a T-cannula at the distal ileum as previously described (Sauer et al., 1983; de Lange et al., 1989). Pigs were housed individually in metabolism pens (1.2 m wide, 1.4 m long and 0.94 m high) made of polyvinylchloride walls and fully slatted plastic mesh flooring. Pens were equipped with a stainless steel wet/dry self-feeder attached to the front wall and a single cup drinker next to the feeder. Pigs had visual contact with neighbouring pigs through a clear window. Room temperature was maintained within the thermo-neutral zone of pigs using a negative pressure ventilation system.

2.3.2. Experimental diets

Pigs were fed 6 mash diets including a basal (460 g/kg wheat and corn starch), 4 diets with 460 g/kg wheat and either *B. juncea* or *B. napus* canola meal at 250 or 500 g/kg, and an N-free diet (Table 1). The N-free diet based on corn starch was used to quantify basal endogenous CP and AA losses (Stein et al., 2006). Diets were supplemented with vitamin and mineral premixes to meet or exceed their requirements (NRC, 2012).

2.3.3. Experimental design and procedures

Pigs were fed the 6 diets in a 6 × 6 Latin square to provide 6 pig observations per diet. Each experimental period was 10 days long with 5 d of adaptation to the test diet, sequentially followed by 2 d of continuous faecal collection, and 3 d of ileal digesta collection. Pigs had free access to water throughout the experiment. Feed allowance was adjusted to 2.8 times the maintenance energy requirement [110 kcal of digestible energy (DE)/kg of $BW^{0.75}$; NRC, 1998] in 2 equal meals at 0730 and 1530 h daily.

Faeces were collected in plastic bags attached to the skin around the anus using a Velcro ring and medical adhesive (Van Kleef et al., 1994). Digesta was collected using plastic bags (contained 15 ml of 5% formic acid) attached to the opened barrel of the cannula by an elastic band. Faeces and digesta were collected from 07h30 to 16h00 when plastic bags were full or at least every 45 minutes. Faeces or digesta were pooled for each pig observation, frozen at -20°C; later homogenised, sub-sampled, freeze-dried and ground.

2.3.4. Chemical analyses

Test ingredients, diets, freeze-dried digesta and faeces were ground through a 1-mm screen in a centrifugal mill (model ZM200, Retsch GmbH, Haan, Germany). Test ingredients were analysed for moisture (method 930.15; AOAC, 2006), CP (N × 6.25; method 988.05; AOAC, 2006), ether extract (hexane, method 920.39, AOAC, 1990), ADF inclusive of residual ash (method 973.18, AOAC, 2006), NDF (Holst, 1973), ash (method 942.05; AOAC, 2006), starch (assay kit STA-20; Sigma, St. Louis, MO, USA), calcium (method 968.08) and phosphorus (method 946.06). The AA profile in test ingredients, diets and digesta was determined by HPLC (method 982.30E; AOAC, 2006) whereas chemically-available lysine was analysed by spectrophotometry (method 975.44; AOAC, 2006). Gross energy was determined using an adiabatic bomb calorimeter (model 5003, Ika-Werke GMBH & Co.KG, Staufen, Germany) with benzoic acid as a standard. Chromic oxide in diets, digesta and faeces was assessed by spectrophotometry at 440 nm (Fenton and Fenton, 1979). Concentration of VFA in digesta and faeces was determined by gas chromatography (model 3400; Varian, Walnut Creek, CA, USA) using 4-methyl-valeric acid as an internal standard.

2.3.5. Calculations

The apparent ileal digestibility coefficient (CAID) and apparent total tract digestibility coefficient (CATTD) of nutrients in diets were calculated using the index method based on chromic oxide (Cr_2O_3) data (Adeola, 2001). The coefficient of apparent hindgut fermentation (CAHF) was calculated using CAID and CATTD in two formulas:

CAHF (as proportion of GE in diet or ingredient) = CATTD of GE (diet or ingredient) – CAID of GE (diet or ingredient); and

CAHF (as proportion of GE in ileal digesta) = CAHF of GE (as proportion of GE in diet or ingredient)/ (1 – CAID of GE).

For CAID and CATTD of energy of ingredients, the N-free diet fed to each pig was used to calculate CAID and CATTD of wheat and canola meal using the difference method (Stein, 2006). The ratio of corn starch, sugar and canola oil was identical among the 6 test diets to calculate energy digestibility of wheat and canola meal at the 2 inclusion levels. Therefore, measured CAID (and CATTD) of energy in the N-free diet was used to calculate CAID (and CATTD) of energy of wheat and subsequently of canola meal by difference using the mass contribution of each ingredient that supplies energy to the diet (Fan and Sauer, 1995):

CAID (wheat) = [CAID (wheat diet) – (% N-free in diet × CAID (N-free))]/% wheat in wheat diet, and

CAID (canola meal in test diet) = [CAID (test diet) – (% N-free in test diet × CAID (N-free) + % wheat in test diet × CAID (wheat))]/% canola meal in test diet

The DE value of test ingredients was calculated by multiplying its GE content with its CATTD of GE, followed by standardisation to 100 g moisture/kg.

The NE value of ingredients was calculated based on DE values and chemical composition (CP, ether extract, starch and ADF) using the equation developed by Noblet et al. (Noblet et al., 1994) and adopted by NRC (2012):

NE = 0.7 × DE + 1.61 × ether extract + 0.48 × starch – 0.91 × CP – 0.87 × ADF, where NE and DE values are expressed as kcal/kg of DM and the macronutrients as g/kg of DM. The NE values were then converted from kcal/kg of diet as DM basis to MJ/kg of diet standardised to 100 g/kg moisture.

For AA, the basal ileal endogenous loss (I_{end}) of an AA or CP (g/kg of DM intake) was calculated using the N-free diet (Stein et al., 2007):

I_{end}, g/kg of DM intake = [AA or CP in digesta × (Cr_2O_3 in feed / Cr_2O_3 in digesta)]

For diets, the standardized ileal digestibility coefficients (CSID) of each indispensable AA were calculated by correcting the CAID for basal ileal endogenous losses using the equation (Stein et al., 2007):

CSID = [CAID + (IAA_{end} /AA intake)]

For wheat, the CSID of AA was considered the CSID of wheat. For canola meal, the CSID of each AA were calculated by difference using the contribution of ingredient that supplies AA in the diet:

CSID (canola meal in test diet) = [CSID (test diet) – (% AA from wheat × CSID (wheat))]/% AA from canola meal in test diet.

2.3.6. Statistical analyses

Data were analysed using the MIXED procedure of SAS (version 9.4; SAS Inst. Inc., Cary, NC, USA) with individual pig as the experimental unit. Normality and homogeneity of variance of the residual of each variable were confirmed using UNIVARIATE procedure with

'Normal' option and GLM procedure with 'Hovtest = Levene' option, respectively. The 2 × 2 model included canola species (*B. juncea* and *B. napus*) and inclusion level (250 and 500 g/kg) as fixed effects and their interaction. Period and pig were random terms. The N-free diet was used for calculation of basal ileal endogenous loss of AA and CP. The wheat diet served as the basal to calculate digestibility of canola meal and as the control diet when comparing canola meal to wheat grain. An orthogonal contrast compared the 4 canola diets to the wheat diet on digestibility of energy, AA and hindgut fermentation. Means separation was obtained using PDIFF option with Tukey adjustment when interactions were observed. Differences were considered significant if $P < 0.05$ and a trend if $0.05 \leq P < 0.10$. Values are presented as least square means and pooled SEM.

2.4. Results

The *B. junce*a canola meal contained 37% less ADF, 26% less NDF and 1.6% less GE than *B. napus* canola meal (Table 2). The CP content and AA profile was similar in *B. juncea* canola meal and *B. napus* canola meal, but *B. juncea* canola meal contained 9% less total lysine and 10% less chemically-available lysine. Diet CP and chemically-available lysine content increased up to 62% and 74% for *B. juncea* and 61% and 84% for *B. napus*, respectively, compared with the wheat diet (Table 3).

Canola species and inclusion level interacted ($P = 0.018$; Table 4) for diet DE value and interacted ($P = 0.035$) for CATTD of diet GE. Diet DE values were reduced ($P < 0.001$) 3.6% and 6.0% by increasing *B. juncea* and *B. napus* canola meal inclusion from 250 to 500 g/kg, respectively. Diet CATTD of GE and diet DE value were greatest for 250 g *B. juncea*/kg and lowest for 500 g *B. napus*/kg. Increasing dietary inclusion of *B. juncea* and *B. napus* canola meal

reduced ($P < 0.01$) diet CAID of GE. Diet CAHF of GE (proportion of GE in ileal digesta) tended to be greater ($P = 0.051$) for *B. juncea* than for *B. napus* diets. Diet DE value, CAID of GE and CATTD of GE were greater ($P < 0.001$) for wheat than canola meal diets, but CAHF of GE (proportion of GE in diet) was lower ($P < 0.05$) for the wheat diet. Ingredient CAID of GE did not differ. Ingredient CATTD of GE was greater ($P < 0.01$) for *B. juncea* than *B. napus* canola meal. The DE and predicted NE values were thus greater ($P < 0.01$) for *B. juncea* than for *B. napus* canola meal. Ingredient CAHF of GE did not differ. Ingredient CAID and CATTD of GE but not CAHF of GE were greater ($P < 0.001$) for wheat grain than canola meal.

An interaction between canola species and inclusion level was not observed for ingredient CSID (Table 5). The CSID of CP, lysine and chemically-available lysine, threonine and methionine did not differ between *B. juncea* and *B. napus* canola meal and inclusion level. Diets containing canola meal had greater ($P < 0.001$) CSID of lysine, chemically-available lysine and methionine than the wheat diet.

An interaction between canola species and inclusion level was not observed for digesta VFA, except for butyrate (Table 6). Concentration of digesta butyrate was lowest ($P = 0.05$) for pigs fed 500 g *B. juncea*/kg and greatest for pigs fed 250 g *B. napus*/kg. Concentration of digesta acetate, isobutyrate, isovalerate and total VFA was lower ($P < 0.01$) in pigs fed *B. juncea* than *B. napus* canola meal diets. Concentration of digesta VFA was lower ($P < 0.05$) for 500 than 250 g canola meal/kg, except for caproic acid. In faeces, interactions between canola species and inclusion level were observed for faecal acetate ($P = 0.026$) and for total VFA ($P = 0.039$). Concentrations of individual and total VFA in faeces did not differ between pigs fed *B. juncea* and *B. napus* canola meal diets, except for 13% lower valerate and 86% greater caproic acid for

B. juncea than *B. napus* canola meal. Concentration of digesta but not faecal total VFA was greater ($P < 0.01$) for wheat than canola meal diets.

For molar ratio of digesta VFA, an interaction between canola species and inclusion level was not observed, except for butyrate ($P = 0.013$; Table 7). The molar ratio of digesta butyrate was lowest ($P < 0.05$) for pigs fed 500 g *B. juncea*/kg and greatest for pigs fed 250 g *B. napus*/kg. Increasing inclusion of canola meal in diets increased ($P < 0.05$; Table 7) the molar ratio of acetate and caproic acid in digesta by 4.3% and 67% for *B. juncea*, and by 4.8% and 40% for *B. napus*, respectively. Increasing dietary inclusion of canola meal reduced ($P < 0.05$) the ratio of digesta propionate. In faeces, interactions between canola species and inclusion level were observed for molar ratios of isobutyrate ($P = 0.003$), isovalerate ($P = 0.003$), caproic acid ($P = 0.029$) and total VFA ($P = 0.039$). The molar ratio of acetate and caproic acid was greater ($P < 0.01$) and of butyrate and valerate was lower ($P < 0.05$) for *B. juncea* than *B. napus* diets.

2.5. Discussion

2.5.1. Diet formulation

Digestibility of feedstuffs in pigs can be studied in diets including either corn starch or common feedstuffs. In the present study, the feeding of a wheat-based diets not only represented common swine diets in western Canada and northern Europe, but also took into account combined effects of wheat and canola meal on digestibility of energy and AA and hindgut fermentation in growing pigs. Digestibility of canola meal is associated with dietary protein content, dietary fibre originating from the basal energy source in the diet (grain or starch) and animal age (Bell and Keith, 1989). Thus, the study was specifically designed to analyse effects of canola species, inclusion level of canola meal, interaction between canola species and inclusion

level in diets based on wheat, and compared canola meal to wheat for nutrient digestibility and fermentation metabolites.

The experimental design and diet formulation of the present study was unique compared with other studies that solely included test diets and one N-free diet to calculate basal ileal endogenous losses (e.g., Trindade Neto et al., 2012; Woyengo et al., 2016b). First, the ratio of canola oil, sugar and canola oil was identical between the N-free diet and other test diets supporting the calculation of energy digestibility first in wheat and then canola meal using the difference method (Stein et al., 2006). Thereby, the N-free diet served a dual purpose: calculation of CSID of AA and CAID and CATTD of energy of test ingredients. Wheat was included at 460 g/kg in all test diets, except for the N-free diet, while the content of canola oil, sugar and canola oil fluctuated. Therefore, calculation of digestibility of canola meal using the difference method was supported (Adeola, 2001). These formulations provided information on effects of canola meal and its inclusion level on digestibility of energy and AA and hindgut fermentation in growing pigs. The canola × inclusion interaction for energy digestibility that was observed in the present study provided further evidence that are core concept of least-cost feed formulation, i.e., that energy value of individual ingredients add up to be energy value of the diet, might not be valid under all conditions (Zhou et al., 2017).

2.5.2. Energy digestibility

The greater CATTD of GE for *B. juncea* than *B. napus* canola meal could be associated with its lower fibre content and different NSP profile that may favour bacterial activity (Bell et al., 1998; Slominski et al., 2012). *B. juncea* canola meal contains less lignin and polyphenols and more pectic polysaccharides (304 vs. 266 g/kg) than *B. napus* canola meal (Slominski et al., 2012). In addition, the greater content of oligosaccharides (36 vs. 31 g/kg) in *B. juncea* canola

meal than *B. napus* canola meal facilitates fermentation and thereby contributes more energy in the form of VFA (Jia et al., 2012) and might partly explain the greater fermentability of *B. juncea* canola meal compared with *B. napus* canola meal in the present study.

The greater CATTD of ADF (0.67 vs. 0.41) and NDF (0.76 vs. 0.52) for *B. juncea* canola meal than *B. napus* canola meal indicated that *B. juncea* canola meal was fermented better than *B. napus* canola meal (Zhou et al., 2015). Moreover, the CATTD of diet GE and DE value reduced at a greater rate with increased inclusion of *B. napus* canola meal than *B. juncea* canola meal indicating the negative effect of highly-lignified fibre in *B. napus* canola meal on energy digestibility. Consequently, the predicted NE value was greater for *B. juncea* canola meal than *B. napus* canola meal, similar to previous results (Heo et al., 2014; Zhou et al., 2015; Woyengo et al., 2016b).

The predicted NE value of *B. juncea* canola meal in the present study was greater than the value reported by NRC (2012) but lower than the value reported by Woyengo et al. (2016b). The predicted NE value for *B. napus* canola meal was lower than those reported previously (NRC, 2012; Woyengo et al., 2016b). With similar CATTD of energy, lower GE and thus DE value of *B. napus* and *B. juncea* canola meal were lower than reported by NRC (2012) and other authors (Woyengo et al., 2010; Liu et al., 2016; Woyengo et al., 2016b) caused the lower predicted NE value for *B. napus* canola meal.

The reduced CAID of diet energy with increased inclusion of canola meal could be due to the high fibre content of canola meal. It is because fibre, with digestibility coefficients from 0.4 to 0.6, is less digestible than other macronutrients (Noblet et al., 2001). Feed components that are not digested in the small intestine enter the hindgut and can be further degraded through

microbial fermentation and thus contribute to total digestible energy of pigs (Shi and Noblet, 1993).

The lower CAHF of GE as proportion of diet for the wheat diet indicating that wheat contributed less to hindgut fermentation than canola meal. Wheat energy was more digestible in the small intestine than energy in canola meal. The CAHF of GE as proportion of diet represents hindgut fermentation as proportion of the entire diet and the CAHF of GE as proportion of ileal digesta represents fermentability of undigested residue entering the large intestine (Woyengo et al., 2016a). The difference is associated with the lower fibre content and hence increased energy digestion in wheat than in canola meal (Jha et al., 2011). The CAHF of GE as proportion of GE in ileal digesta was greater for *B. juncea* canola than *B. napus* canola meal, indicating greater hindgut fermentation of undigested residue of *B. juncea* canola meal accounting for the greater CATTD of GE for *B. juncea* canola meal than *B. napus* canola meal (Woyengo et al., 2016b).

2.5.3. Crude protein and amino acid digestibility

The SID coefficients of CP and AA did not differ between *B. juncea* and *B. napus* canola meal. The lack of difference indicated that fibre was less of a hindrance to digest AA than energy in canola meal (Berrocoso et al., 2015; Liu et al., 2016); however, SID coefficients of some AA were greater for *B. juncea* canola meal than *B. napus* canola meal in some studies (Trindade Neto et al., 2012; Zhou et al., 2015). Generally, digestibility of AA in *B. juncea* canola meal and *B. napus* canola meal is influenced by canola species, methods of oil extraction and their interactions (Trindade Neto et al., 2012; Woyengo et al., 2016b).

Despite similar SID coefficients of AA of the two canola meals in the present study, SID content of methionine, tryptophan and chemically-available lysine was lower for *B. juncea* canola meal than *B. napus* canola meal (data not shown). This difference was opposite to the

expected greater SID content of *B. juncea* canola meal due its lower fibre content than *B. napus* canola meal. However, the lower content of AA in *B. juncea* canola meal than *B. napus* canola meal prevented that equal SID content of AA was reached in the present study.

2.5.4. Volatile fatty acids

Microbial VFA production is influenced by various factors including dietary fibre, passage rate, diversity of intestinal microbiota population and type and rate of VFA absorption (Macfarlane and Macfarlane, 2003). The VFA such as acetate, butyrate and propionate are derived from microbial carbohydrate fermentation. The breakdown of protein in the hindgut produces VFA, branched-chain fatty acids (BCFA) including isobutyrate, isovalerate, valerate and caproic acid, gases, ammonia, phenols, indoles and amines (Cummings et al., 1991). The intestinal fermentability of canola meal diets was expressed as concentrations of VFA and molar ratios of individual VFA. The former indicated total amount of fermentation whereas the latter provided a better evaluation of fermentation characteristics of the substrates entering the hindgut (Macfarlane and Macfarlane, 2003). The greater total VFA concentration in faeces than in ileal digesta indicates that microbial fermentation started at the end of the small intestine and increased in the hindgut (Graham et al., 1986; Jensen and Jørgensen, 1994).

Acetate was the major VFA both in digesta and faeces. The greater ratio of acetate to propionate and butyrate in ileal digesta than in faeces indicated that fermentation of soluble NSP such as pectin started in the small intestine (Zacharias et al., 2004). The lower concentrations of individual and total VFA in the ileal digesta for *B. juncea* than *B. napus* canola meal could be attributed to the lower fibre content in *B. juncea* canola meal (Stanogias and Pearce, 1985). The amount of VFA is correlated to the ileal recovery of dietary NSP at the ileum (Bach Knudsen and Hansen, 1991). The ileal VFA declined with increasing dietary inclusion of either canola

meal, a decline that might be associated to increased less-fermentable fibre content in these diets. The decreased digesta concentration and molar ratio of butyrate with increasing inclusion of canola meal could be associated with reduction in starch content of these diets (Topping and Clifton, 2001).

The CAHF of GE as proportion of ileal digesta was greater for *B. juncea* canola meal, indicating that undigested residue introduced into the hindgut was more fermentable for *B. juncea* canola meal than for *B. napus* canola meal. Fibre described as NSP in the diet plays the greatest role in hindgut fermentation (Anguita et al., 2006). Consequently, the extent of fermentation was lower for *B. napus* canola meal that contained more lignified fibre (Bach Knudsen and Hansen, 1991; Bach Knudsen, 2001).

Total VFA concentration in faeces was influenced by interactions between canola species and inclusion level, and was the lowest for 500 g *B. napus*/kg diet and the greatest for 250 g *B. napus*/kg diet. The interaction can be attributed to increased lignified fibre in *B. napus* canola meal that further decreased fermentation. The dietary protein content also increased with increasing inclusion of canola meal; therefore, more dietary protein was available for microbial fermentation. Protein fermentation occurs in the distal large intestine where pH decreases due to depletion of carbohydrate (Windey et al., 2012) and might be illustrated by increased concentration of BCFA. Ratios of isobutyrate and isovalerate were greater for 500 g *B. napus*/kg diet and 250 g *B. juncea*/kg diet indicating enhanced protein fermentation. However, increased fermentation of carbohydrate for 500 g *B. juncea*/kg diet reduced ratios of isobutyrate and isovalerate. Similarly, production of isobutyrate decreased when more fermentable carbohydrates were available (Marfarlane et al., 1992). However, the concentration of VFA measured from

ileal digesta or faeces may not represent VFA production in the intestinal lumen because 95% of VFA are quickly absorbed by the host (Cummings et al., 1991; Topping and Clifton, 2001).

2.6. Conclusions

Based on the 2 samples evaluated, *B. juncea* contained less fibre than *B.* napus canola meal resulting in greater fermentability and ATTD of energy, but digestibility of AA did not differ. Increasing dietary inclusion of canola meal from 250 to 500 g/kg reduced digestibility of energy but did not affect standardised ileal digestibility of AA. Fermentability of *B. napus* canola meal but not *B. juncea* canola meal decreased in the pig intestine with increased dietary inclusion. Hence, yellow-seeded *B. juncea* canola meal had greater DE value, but similar amino acid digestibility as conventional black-seeded *B. napus* canola meal. Its use will increase flexibility in swine feed formulation, increase meal utilization of a canola species grown in marginal prairie land and may limit proteolytic fermentation in the pig intestine.

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Table 2.1. Ingredient composition (g/kg diet as-fed) of the experimental diets.

Ingredient	Wheat	Canola meal (g/kg diet)				N-free
		B. napus		*B. juncea*		
		250	500	250	500	
Corn starch	429	214	-	214	-	780
B. napus canola meal	-	250	500	-	-	-
B. juncea canola meal	-	-	-	250	500	-
Wheat	460	460	460	460	460	-
Sugar	55	27	-	27	-	100
Cellulose[a]	-	-	-	-	-	40
Canola oil	16	8	-	8	-	30
Limestone	12	12	12	12	12	9
Mono-dicalcium phosphate	9	9	9	9	9	17
Salt	4	4	4	4	4	4
Vitamin premix[b]	5	5	5	5	5	5
Mineral premix[c]	5	5	5	5	5	5
KCO_3,56% K	-	-	-	-	-	4
MgO, 58% Mg	-	-	-	-	-	1
Chromic oxide	5	5	5	5	5	5

[a] Solka floc, International Fiber Corp., North Tonawanda, NY, USA.

[b] Provided the following per kilogram of diet: vitamin A, 8,250 IU; vitamin D_3, 825 IU; vitamin E, 40 IU; niacin, 35 mg; D-pantothenic acid, 15 mg; riboflavin, 5 mg; menadione, 4 mg; folic acid, 2 mg; thiamine, 1 mg; D-biotin 0.2 mg and vitamin B_{12}, 0.025 mg.

[c] Provided the following per kilogram of diet: Zn, 100 mg as $ZnSO_4$; Fe, 80 mg as $FeSO_4$; Cu, 50 mg as $CuSO_4$; Mn, 25 mg as $MnSO_4$; I, 0.5 mg as $Ca(IO_3)_2$ and Se, 0.1 mg as Na_2SeO_3.

Table 2.2. Analysed nutrient composition of *B. juncea* and *B. napus* canola meal and wheat.

Item (g/kg, as-is basis)	Wheat	Canola meal	
		B. napus	*B. juncea*
Moisture	116	84	79
CP	124	386	386
Gross energy, MJ/kg	16.3	18.3	18.0
Starch	511	0	15
Ether extract	24	26	24
Ash	18	80	77
Neutral detergent fibre	140	258	190
Acid detergent fibre	27	190	119
Phosphorus	4	12	13
Calcium	1	7	7
Indispensable amino acids			
Arginine	6.5	24.1	26.4
Histidine	3	10.7	10.5
Isoleucine	4.8	16.3	15.8
Leucine	9.1	28.1	29.0
Lysine	4.2	22.1	20.3
Chemically-available lysine	4.0	21.0	19.0
Methionine	2.1	7.9	7.3
Phenylalanine	6.2	16.2	16.4
Threonine	4.0	16.4	16.8
Tryptophan	1.6	5.0	4.1
Valine	6.1	21.2	19.9
Dispensable amino acids			
Alanine	4.9	17.4	17.9
Aspartate	7.0	27.9	29.9
Cysteine	2.9	9.6	7.9
Glutamate	37.7	65.8	64.9
Glycine	5.6	20.1	20.4
Proline	12.4	22.7	21.8
Serine	5.3	13.4	14.6
Tyrosine	3.7	11.3	11.8

Table 2.3. Analysed nutrient composition of the experimental diets.

Item (g/kg diet as-fed)	Wheat	Canola meal (g/kg diet)				N-free
		B. napus		*B. juncea*		
		250	500	250	500	
Moisture	100	101	107	101	105	80
Crude protein	56	153	247	154	250	4
Ash	33	54	73	54	72	30
Ether extract	18	18	18	17	17	28
Gross energy, MJ/kg	15.9	16.3	16.5	16.2	16.4	15.7
Indispensable amino acids						
Arginine	2.5	8.9	15.8	9.4	15.9	0.1
Histidine	1.2	4.0	7.0	4.0	6.5	0.0
Isoleucine	1.9	6.2	10.6	6.3	10.4	0.1
Leucine	3.6	11.0	19.1	11.2	18.4	0.2
Lysine	1.6	7.4	13.6	6.9	12.0	0.1
Chemically-available lysine	1.5	7.0	12.9	6.5	11.3	0.1
Methionine	0.8	2.9	5.0	2.6	4.4	0.0
Phenylalanine	2.4	6.7	11.3	6.7	10.8	0.1
Threonine	1.5	6.0	10.8	5.9	9.9	0.1
Tryptophan	0.7	1.9	3.0	1.8	2.8	0.4
Valine	2.4	7.9	13.7	7.8	13.0	0.1
Dispensable amino acids						
Alanine	2.0	6.6	11.6	6.7	11.1	0.1
Aspartate	2.9	10.3	18.1	10.8	18.0	0.1
Cysteine	1.1	3.6	6.3	3.2	5.1	0.0
Glutamate	14.7	33.1	52.9	32.8	49.0	0.2
Glycine	2.2	7.6	13.3	7.6	12.6	0.1
Proline	4.8	11.3	17.9	10.7	16.0	0.2
Serine	2.0	5.7	9.9	5.7	9.1	0.1
Tyrosine	1.2	4.3	7.7	4.4	7.5	0.1

Table 2.4. Energy digestibility, fermentability and energy value of wheat and canola meal diets and ingredients (standardised to 100 g moisture/kg).

Item[a]	Wheat	Canola meal (g/kg diet)				SEM[b]	*P*-value[c]			
		B. napus		*B. juncea*			Canola	Inclusion	Canola × Inclusion	Wheat vs. Canola
		250	500	250	500					
Diet										
DE, MJ/kg	14.4	13.4^{xy}	12.6^{w}	13.7^{yz}	13.2^{x}	0.12	< 0.001	< 0.001	0.018	< 0.001
Energy digestibility										
CAID	0.82	0.71	0.60	0.70	0.64	0.03	0.634	0.003	0.253	< 0.001
CATTD	0.91	0.82^{y}	0.76^{w}	0.84^{z}	0.80^{x}	0.01	< 0.001	< 0.001	0.035	< 0.001
CAHF of GE (proportion of GE in ileal digesta)	0.46	0.37	0.38	0.45	0.44	0.05	0.051	0.932	0.669	0.199
CAHF of GE (proportion of GE in diet)	0.09	0.11	0.15	0.15	0.16	0.03	0.339	0.203	0.435	0.017
Ingredient										
DE, MJ/kg	15.0	10.5	11.2	11.8	12.4	0.66	0.005	0.080	0.867	< 0.001
NE, MJ/kg	10.5	7.14	7.63	8.09	8.50	0.46				
Energy digestibility										
CAID	0.77	0.35	0.45	0.45	0.52	0.06	0.088	0.099	0.784	< 0.001
CATTD	0.90	0.64	0.62	0.70	0.70	0.04	0.001	0.447	0.389	< 0.001
CAHF of GE (proportion of GE in ileal digesta)	0.53	0.42	0.31	0.41	0.38	12.1	0.696	0.313	0.363	0.118
CAHF of GE (proportion of GE in ingredient)	0.13	0.29	0.17	0.25	0.18	0.07	0.687	0.091	0.567	0.089

[a] CAID = coefficient of apparent ileal digestibility; CATTD = coefficient of apparent total tract digestibility; CAHF = coefficient of apparent hindgut fermentation. NE values were calculated based on DE values and chemical composition using equation (Noblet at al., 1994; NRC, 2012).

[b] 6 pig observations per diet.

[c] Canola (*B. napus* vs. *B. juncea*), Inclusion (250 vs. 500 g/kg of *B. napus* or *B. juncea*), Canola × Inclusion (interaction between canola species and inclusion level), Wheat vs. Canola (wheat vs. 4 canola meal).

[w, x, y, z] For observed Canola × Inclusion interactions ($P < 0.05$), means without a common superscript differ ($P < 0.05$).

Table 2.5. Standardised ileal digestibility coefficients (CSID) of CP and amino acids of the test ingredients.

Item	Wheat	Canola meal (g/kg diet)				SEM[a]	*P*-value[b]			
		B. napus		*B. juncea*			Canola	Inclusion	Canola × Inclusion	Wheat vs. Canola
		250	500	250	500					
CP	0.77	0.82	0.80	0.84	0.83	0.05	0.403	0.687	0.785	0.125
Indispensable amino acids										
Arginine	0.78	0.96	0.92	0.99	0.93	0.04	0.085	0.001	0.167	< 0.001
Histidine	0.84	0.91	0.88	0.93	0.89	0.03	0.286	0.050	0.705	0.031
Isoleucine	0.80	0.85	0.84	0.86	0.84	0.03	0.803	0.465	0.945	0.126
Leucine	0.84	0.86	0.85	0.85	0.84	0.03	0.750	0.579	0.925	0.875
Lysine	0.53	0.81	0.84	0.85	0.86	0.05	0.421	0.369	0.713	< 0.001
Methionine	0.81	0.91	0.90	0.90	0.90	0.03	0.660	0.565	0.893	< 0.001
Phenylalanine	0.84	0.87	0.85	0.86	0.85	0.03	0.964	0.452	0.901	0.637
Threonine	0.78	0.83	0.82	0.81	0.82	0.05	0.873	0.927	0.776	0.352
Tryptophan	0.86	0.86	0.83	0.85	0.84	0.04	0.900	0.442	0.605	0.649
Valine	0.81	0.83	0.82	0.83	0.83	0.04	0.863	0.908	0.887	0.571
Dispensable amino acids										
Alanine	0.71	0.85	0.85	0.86	0.85	0.05	0.871	0.903	0.943	0.001
Aspartate	0.73	0.86	0.84	0.86	0.85	0.04	0.663	0.515	0.892	0.003
Cysteine	0.88	0.84	0.80	0.79	0.78	0.04	0.281	0.370	0.625	0.036
Glutamate	0.91	0.89	0.87	0.88	0.86	0.02	0.607	0.199	0.933	0.050
Glycine	0.65	0.93	0.89	0.96	0.88	0.09	0.809	0.062	0.549	0.001
Serine	0.83	0.83	0.84	0.83	0.84	0.05	0.942	0.800	0.973	0.872
Tyrosine	0.76	0.88	0.88	0.90	0.89	0.04	0.648	0.815	0.965	< 0.001
Chemically-available lysine	0.52	0.87	0.85	0.86	0.87	0.06	0.907	0.970	0.605	< 0.001

[a] 6 pig observations per diet.

[b] Canola (*B. napus* vs. *B. juncea*), Inclusion (250 vs. 500 g/kg of *B. napus* or *B. juncea*), Canola × Inclusion (interaction between canola species and inclusion level), Wheat vs. Canola (wheat vs. 4 canola meal).

[x, y] For observed Canola × Inclusion interactions ($P < 0.05$), means without a common superscript differ ($P < 0.05$).

Table 2.6. Concentration of volatile fatty acids (VFA) and total VFA (µmol/g) of wet digesta and faeces.

Item	N-free	Wheat	Canola meal (g/kg diet)				SEM[a]	*P* value[b]			
			B. napus		*B. juncea*			Canola	Inclusion	Canola × Inclusion	Wheat vs. Canola
			250	500	250	500					
Digesta											
Acetate	9.81	18.9	21.7	16.0	16.3	11.2	1.73	< 0.001	< 0.001	0.782	0.062
Propionate	1.21	1.47	0.97	0.62	0.92	0.49	0.20	0.386	< 0.001	0.745	< 0.001
Butyrate	0.40	2.35	1.47^{z}	0.39^{x}	0.79^{y}	0.23^{x}	0.36	< 0.001	< 0.001	0.029	< 0.001
Isobutyrate	0.04	0.06	0.07	0.03	0.05	0.02	0.01	0.005	< 0.001	0.323	0.054
Isovalerate	0.07	0.08	0.13	0.07	0.09	0.04	0.02	0.007	< 0.001	0.583	0.858
Valerate	0.09	0.07	0.04	0.02	0.03	0.02	0.01	0.336	0.041	0.901	< 0.001
Caproic acid	0.03	0.03	0.02	0.02	0.02	0.02	0.01	0.199	0.353	0.972	< 0.001
Total VFA	11.7	22.9	24.4	17.2	18.2	12.1	2.03	< 0.001	< 0.001	0.655	0.002
Faeces											
Acetate	91.6	99.5	102^{z}	88.3^{y}	93.7^{yz}	103^{z}	7.47	0.443	0.658	0.026	0.633
Propionate	11.9	30.3	40.1	33.9	35.2	36.4	2.94	0.542	0.178	0.065	0.012
Butyrate	9.79	19.2	29.5	22.5	23.7	23.9	3.01	0.253	0.065	0.060	0.021
Isobutyrate	1.82	3.75	3.88	3.83	3.88	3.65	0.32	0.697	0.463	0.625	0.814
Isovalerate	1.78	5.55	5.38	5.34	5.49	5.01	0.47	0.756	0.361	0.436	0.511
Valerate	1.38	3.95	4.67	4.53	3.87	4.10	0.54	0.023	0.927	0.517	0.426
Caproic acid	0.31	0.34	0.19	0.25	0.48	0.34	0.10	0.005	0.561	0.126	0.708
Total VFA	119	163	185^{z}	159^{y}	166^{yz}	177^{yz}	13.6	0.981	0.316	0.039	0.403

[a] 6 pig observations per diet.

[b] Canola (*B. napus* vs. *B. juncea*), Inclusion (250 vs. 500 g/kg of *B. napus* or *B. juncea*), Canola × Inclusion (interaction between canola species and inclusion level), Wheat vs. Canola (wheat vs. 4 canola meal).

[x, y, z] For observed Canola × Inclusion interactions ($P < 0.05$), means without a common superscript differ ($P < 0.05$).

Table 2.7. Molar ratio of volatile fatty acids (VFA) and total VFA (µmol/g) of wet digesta and faeces.

Item	N-free	Wheat	Canola meal (g/kg diet)				SEM[a]	*P* value[b]			
			B. napus		*B. juncea*			Canola	Inclusion	Canola × Inclusion	Wheat vs. Canola
			250	500	250	500					
Digesta											
Acetate	86.5	84.4	89.0	93.3	88.2	92.0	1.42	0.225	< 0.001	0.761	< 0.001
Propionate	8.81	7.02	4.53	3.68	7.14	5.10	1.04	0.004	0.036	0.388	0.022
Butyrate	2.62	7.55	5.38^{z}	2.12^{x}	3.54^{y}	1.91^{x}	0.72	0.002	< 0.001	0.013	< 0.001
Isobutyrate	0.33	0.21	0.28	0.20	0.25	0.22	0.05	0.916	0.125	0.530	0.500
Isovalerate	0.63	0.32	0.49	0.41	0.48	0.39	0.09	0.827	0.191	0.100	0.080
Valerate	0.78	0.34	0.18	0.15	0.25	0.21	0.07	0.214	0.555	0.865	0.014
Caproic acid	0.33	0.15	0.10	0.14	0.12	0.20	0.04	0.137	0.034	0.482	0.875
Total VFA	11.7	22.9	24.4	17.2	18.2	12.1	2.03	< 0.001	< 0.001	0.655	0.002
Faeces											
Acetate	76.8	62.1	55.2	55.6	56.7	59.0	1.13	0.002	0.054	0.196	< 0.001
Propionate	10.4	18.3	21.5	21.3	21.2	20.5	0.58	0.239	0.230	0.519	< 0.001
Butyrate	8.20	10.8	15.5	14.2	13.8	13.1	0.86	0.021	0.095	0.598	< 0.001
Isobutyrate	1.56	2.46	2.14^{y}	2.42^{z}	2.35^{z}	2.06^{y}	0.16	0.436	0.882	0.003	0.091
Isovalerate	1.55	3.68	2.97^{yz}	3.39^{z}	3.32^{z}	2.81^{y}	0.26	0.461	0.712	0.003	0.009
Valerate	1.24	2.41	2.58	2.88	2.35	2.31	0.20	0.001	0.262	0.114	0.441
Caproic acid	0.28	0.22	0.11^{y}	0.16^{y}	0.28^{z}	0.18^{y}	0.05	0.004	0.465	0.029	0.331
Total VFA	119	163	185^{z}	159^{y}	166^{yz}	177^{yz}	13.6	0.981	0.316	0.039	0.403

[a] 6 pig observations per diet.

[b] Canola (*B. napus* vs. *B. juncea*), Inclusion (250 vs. 500 g/kg of *B. napus* or *B. juncea*), Canola × Inclusion (interaction between canola species and inclusion level), Wheat vs. Canola (wheat vs. 4 canola meal).

[x, y, z] For observed Canola × Inclusion interactions ($P < 0.05$), means without a common superscript differ ($P < 0.05$).

CHAPTER 3 – The effect of feeding increasing inclusion of extruded *Brassica juncea* canola expeller on growth performance and nutrient digestibility in weaned pigs

3.1. Abstract

Expellers contain more dietary energy than meals to support growth performance of young pigs. The feeding value of extruded *Brassica (B.) juncea* canola expeller was evaluated by feeding 240 weaned pigs (initial body weight 7.6 kg), starting 1 week after weaning at 19 days of age. The extruded *B. juncea* expeller contained (as is) 344 g crude protein, 15.7 g chemically-available lysine (Lys), 169 g ether extract, 127 g acid detergent fibre, 195 g neutral detergent fibre/kg and 11 μmol/g total glucosinolates. Pigs were fed 5 pelleted wheat-based diets for two growth phases: Phase 1, days 0-14; and Phase 2, days 15-35. Diets including 0, 60, 120, 180 and 240 g extruded *B. juncea* expeller/kg were formulated to provide 10.0 and 9.7 MJ net energy (NE)/kg and 1.17 and 1.06 standardised ileal digestible (SID) Lys/MJ NE for Phase 1 and 2 diets, respectively. The extruded *B. juncea* expeller substituted soybean meal. Diets were balanced for NE by decreasing canola oil inclusion from 55 to 29 and 26 to 0 g/kg for Phase 1 and 2, respectively; and for amino acids by increasing crystalline amino acids. Increasing dietary inclusion of extruded *B. juncea* expeller linearly reduced (P<0.001) apparent total tract digestibility of dry matter, gross energy and crude protein and decreased diet digestible energy values in both phases. For day 0-35, increasing inclusion of extruded *B. juncea* expeller did not affect feed efficiency, but quadratically increased average daily feed intake (ADFI; P<0.001) and average daily gain (ADG, P<0.01), which corresponded with a quadratic increase (P<0.01) in intake of NE and SID Lys. On day 35, pigs fed 60, 120, 180 and 240 g extruded *B. juncea* expeller/kg were 1.1, 1.5, 1.5 and 1.1 kg heavier (P<0.05), respectively, than control pigs. Feed energy values may explain the achieved performance. For diet formulation, we used 22.46 MJ

NE/kg for canola oil (NRC, 1998) instead of the more recent 31.63 MJ NE/kg (NRC, 2012). Using the revised NE value, calculated diet NE values (as fed) decreased from 10.55 to 10.30 in Phase 1 and from 9.92 to 9.71 MJ NE/kg in Phase 2 diets for pigs fed 0 to 240 g extruded *B. juncea* expeller/kg. In conclusion, reduced diet NE value coincided with increased NE and SID Lys intake that consequently increased ADG. The linear increase of ADFI and ADG may have been curved at 240 g extruded *B. juncea* expeller/kg by increased dietary glucosinolates intake that prevented further increases in ADFI.

Keywords: *Brassica juncea*; Canola expeller; Digestibility; Glucosinolate; Performance; Pig

3.2. Introduction

Inclusion of canola meal (CM) in swine diets may increase flexibility in feed formulation and provide opportunities to reduce feed cost. A co-product of canola seed, CM is globally ranked as the second protein feedstuff after soybean meal. The low energy value of solvent-extracted *Brassica (B.) napus* CM is due to its relatively high fibre and low ether extract content (Newkirk, 2009).

Yellow-seeded *B. juncea* has a thinner seed coat and thus lower fibre content (Khajali and Slominski, 2012). Consequently, digestible energy (DE) and net energy (NE) values were greater for *B. juncea* CM than *B. napus* CM (Le et al., 2012). However, *B. juncea* CM contains double the glucosinolates content than *B. napus* CM, with a great share of gluconapin that tastes bitter and reduced the feed intake of young pigs fed up to 240 g *B. juncea* CM/kg (Landero et al., 2013). Oil extraction reduces the energy value of CM. Expeller-pressed CM contains more residual oil and thus more DE and ME than solvent-extracted CM (Woyengo et al., 2010).

Extrusion of canola seed prior to pressing may increase the feed value of the expeller (Huang et al., 1995; Liang et al., 2002). Extruded canola expeller may contain more ether extract (170 g/kg) than expeller-pressed CM (120 g/kg) or solvent-extracted CM (3 g/kg; Beltranena and Zijlstra, 2011). Hence, extruded *B. juncea* expeller might be valuable for swine feeding; however, its feeding value has not been studied.

Our hypothesis was that weaned pigs fed diets with increasing inclusion of extruded *B. juncea* expeller formulated to equal NE and standardised ileal digestibility (SID) amino acid content would have similar nutrient digestibility and performance. The objectives were to determine the apparent total tract digestibility coefficients (CATTD) of gross energy (GE), crude protein (CP) and dry matter (DM) and DE and calculated NE values of diets; and evaluate the dose response effect of feeding weaned pigs diets including 0, 60, 120, 180 or 240 g extruded *B. juncea* expeller/kg on growth performance.

3.3. Materials and methods

3.3.1. Experimental design and diets

The animal care and use was approved by the University of Alberta Animal Care and Use Committee for Livestock, and followed principles established by the Canadian Council on Animal Care (CCAC, 2009). The study was carried out at the Swine Research and Technology Centre, University of Alberta (Edmonton, Alberta, Canada).

In total, 240 crossbred pigs (Duroc × Large White/Landrace F_1; Hypor, Regina, SK, Canada) were weaned at 19 ± 1 days of age. Pigs were selected based on body weight (BW; 7.6 ± 0.85 kg) and average daily weight gain for the first 7 days after weaning. Pigs were sorted

within gender into light and heavy BW. Two barrows and two gilts (4 pigs/pen) with light and heavy BW were randomly assigned to one of 60 pens.

Pigs were fed a commercial starter diet for 7 days post-weaning before the 2-growth phase feeding study: Phase 1 lasted 14 days and subsequently Phase 2 lasted 21 days. For both feeding phases, 5 wheat-based diets contained 0 (control), 60, 120, 180 or 240 g extruded *B. juncea* expeller/kg in substitution for soybean meal (Table 1). Canola seed was sourced from southern Saskatchewan, Canada and then extruded (at 90^{o}C, flow rate 1050 kg/h, model X155, Wenger, Sabetha, KS, USA) before pressing (at 110^{o}C, flow rate 600 kg/h, model ME-200, Anderson International Corp., Stow, OH, USA) at Apex Nutri-Solutions Inc. (Edberg, AB, Canada). Diets without antimicrobials or growth promoters were formulated to provide 10.0 MJ NE/kg and 1.17 g SID lysine (Lys)/MJ NE for Phase 1 and 9.7 MJ NE/kg and 1.06 g SID Lys/MJ NE for Phase 2. Other amino acids were formulated as an ideal ratio to Lys using established NE and SID amino acid values (NRC, 1998). Acid-insoluble ash (Celite 281; World Minerals, Santa Barbara, CA, USA) was added as indigestible marker at 8 g/kg in diets. Diets were mixed and steam pelleted at 70°C (70hp; California Pellet Mill, Crawfordsville, IN, USA). Pellet sizes were 4.5 and 3.5 mm in diameter; 6-10 and 6-12 mm in length for Phase 1 and Phase 2 diets, respectively.

The 5 dietary treatments were randomly allocated to pens of pigs in a randomised complete block design with 60 pens in 3 nursery rooms filled two weeks apart, for 12 replicate pens per treatment. Within room, blocks including all five dietary treatments were formed across the ventilation gradient. Pens (1.1 × 1.5 m) were equipped with polyvinyl chloride partitions, a 4-space dry feeder (model N4-424; Crystal Springs Hog Equipment, MB, Canada), a nipple drinker and plastic slatted flooring. Room temperature was maintained within the thermo-neutral zone of

pigs, using a negative pressure ventilation system, with a 12-h light (0600-1800h) and 12-h dark cycle. Pigs had free access to feed and water during the trial.

Individual pigs, feed added and leftover were weighed weekly to calculate average daily feed intake (ADFI), average daily gain (ADG) and feed efficiency (G:F) for the pen. Freshly-voided faeces were collected from 0800 to 1600 h by grab sampling from pen floors on days 12, 13 and 33, 34. Faeces were pooled by pen and stored at -20°C. Faeces were then thawed, homogenised, sub-sampled and freeze-dried.

3.3.2. Chemical analyses

Samples of extruded *B. juncea* expeller, diets and freeze-dried faeces were ground through a 1-mm screen in a centrifugal mill (model ZM200, Retsch GmbH, Haan, Germany). The extruded *B. juncea* expeller was analysed for CP (method 984.13A-D), total dietary fibre (method 985.29), acid detergent fibre (ADF) inclusive of residual ash (method 973.18), ash (method 942.05), starch (assay kit STA-20; Sigma, St. Louis, MO, USA), calcium (method 968.08), phosphorus (method 946.06), amino acids (method 982.30E), chemically-available Lys (method 975.44) as described by AOAC (2006) and neutral detergent fibre (NDF; Holst, 1973). Glucosinolates in extruded *B. juncea* expeller were analysed by gas chromatography (Daun and McGregor, 1981) at POS Bio-Sciences, Saskatoon, SK, Canada. Diets were analysed for DM (method 930.15, AOAC, 2006), GE using an adiabatic bomb calorimeter (model 5003; Ika-Werke GMBH & Co. KG, Staufen, Germany), CP (N × 6.25; method 988.05; AOAC, 2006), ether extract, starch, crude fibre, ADF, NDF, amino acids, chemically-available Lys, and acid-insoluble ash (Vogtmann et al., 1975 modified by Newkirk et al., 2003). Faeces were analysed for DM, CP, GE and acid-insoluble ash. Based on results of chemical analyses, CATTD of DM, GE and CP and DE values were calculated using the acid-insoluble ash concentration of faeces relative to

feed by the indicator method (Adeola, 2001). The NE value of diets was calculated based on their DE value and chemical composition (CP, ether extract, starch and ADF) using equation 5 developed by Noblet et al. (1994) and adopted by NRC (2012).

NE = 0.7 DE + 1.61 ether extract + 0.48 starch - 0.91 CP - 0.87 ADF,

where NE and DE values are expressed as kcal/kg of DM and the macronutrients as g/kg of DM.

3.3.3. Statistical analyses

Data were analysed using the MIXED procedure of SAS (version 9.2, SAS Inst. Inc., Cary, NC, USA) with pen as the experimental unit. The fixed effect of inclusion level of extruded *B. juncea* expeller in diets and the random effect of block were included in the statistical model. Growth performance data were analysed as repeated measures with week as the repeated term. Initial BW was included as a covariate to analyse growth performance. Two single-degrees of freedom orthogonal contrasts were used to test the linear or quadratic effects of increasing extruded *B. juncea* expeller inclusion. To test the hypothesis, $P<0.05$ was considered significant.

3.4. Results

The extruded *B. juncea* expeller contained 344 g CP/kg on as is basis with 17.2 g Lys/kg of which 15.7 g/kg was chemically-available (Table 2). Total glucosinolate content was 10.9 µmol/g, including 9.66 µmol 3-butenyl (gluconapin)/g. The Phase 1 and Phase 2 diets including 240 g extruded *B. juncea* expeller/kg contained 5 and 3 g/kg more crude fibre, 15 and 17 g/kg more ADF and 24 and 34 g/kg more NDF, respectively, than the control diets (Table 1).

Increasing dietary inclusion of extruded *B. juncea* expeller linearly reduced ($P<0.001$) the CATTD of DM, CP and GE of both Phase 1 and Phase 2 diets (Table 3). Increasing dietary

inclusion of extruded *B. juncea* expeller linearly decreased (P<0.001) diet DE and calculated NE values for Phase 1 and Phase 2 diets.

For the entire experiment (days 0-35), increasing dietary inclusion of extruded *B. juncea* expeller up to 240 g/kg quadratically increased (P<0.01) ADFI and ADG (Table 4). Overall, increasing inclusion of extruded *B. juncea* expeller also quadratically increased (P<0.01) NE intake and linearly (P<0.01) and quadratically (P<0.001) increased calculated SID Lys intake. Increasing inclusion of extruded *B. juncea* expeller linearly increased (P<0.01) G:F for days 8-14 and 15-21, quadratically decreased (P<0.01) G:F for days 22-28, but did not affect G:F for entire trial. Increasing dietary inclusion of extruded *B. juncea* expeller linearly and quadratically increased (P<0.05) final BW of pigs. Specifically, final BW was 24.4, 25.5, 25.9, 25.9 and 25.5 kg for pigs fed 0, 60, 120, 180 and 240 g extruded *B. juncea* expeller/kg, respectively.

3.5. Discussion

Yellow-seeded *B. juncea*, a novel canola species, is the third *Brassica* oilseed after black-seeded *B. napus* and *B. rapa* recognized in Canada (Canadian Grain Commission, 2013). The *B. juncea* canola is primarily intended to grow in the Brown and Dark Brown soil zones of the lower precipitation and warmer regions of the North American Great Plains (Woods et al., 1991; Gan et al., 2007). As a crop, *B. juncea* is more tolerant to heat and drought stress and more resistant to diseases than *B. napus*. Thus, *B. juncea* may expand canola production where conventional *B. napus* productivity is limited (Miller et al., 2003).

Canola seed contains approximately 420-450 g oil/kg that is commonly extracted by solvents after expeller pressing (Newkirk, 2009; Khajali and Slominski, 2012). Greater residual oil content remains in the expeller if the oil is not solvent-extracted. Residual oil content in the

expeller is influenced by: 1) seed maturity, 2) single or double pressing in tandem of seed, and 3) processing conditions such as seed moisture, conditioning temperature, screw speed and compression rate (Spragg and Mailer, 2007; Newkirk, 2009). The ether extract content of extruded *B. juncea* expeller was 169 g/kg as-is for the present study, greater than that of expeller-pressed CM fed in previous studies: 111 g/kg (Woyengo et al., 2010), 130 g/kg (Seneviratne et al., 2011a) and 103 g/kg (Landero et al., 2012). Thus, inclusion of high residual oil extruded *B. juncea* expeller in nursery diets may provide more dietary energy to meet the energy requirement of young pigs.

Extrusion prior to pressing may increase oil recovery (Nelson et al., 1987). During extrusion, the feedstock is gradually compressed by one or two screws rotating inside a cylindrical barrel (Serrano, 1997) with decreasing space between the screw and barrel wall together with a reduced helix angle. Consequently, shear force and heat generated disrupt cell walls, denature protein, emulsify fat and reduce anti-nutritional factors. Thus, amino acid and lipid digestibility and fibre fermentability of extruded products is increased (Cheftel, 1986; Camire, 1991). Extrusion prior to expeller pressing may thus increase nutrient digestibility.

In the present study, reduced diet nutrient digestibility with increasing dietary inclusion of extruded *B. juncea* expeller coincided with increased content of fibre that hinders digestion in pigs (Bell, 1984; Fernandez and Jorgensen, 1986). Similarly, weaned pigs fed up to 200 g solvent-extracted or expeller-pressed *B. napus* CM/kg or up to 240 g solvent-extracted *B. juncea* CM/kg also had reduced diet nutrient digestibility (Landero et al., 2011, 2012, 2013). Feeding weaned pigs the air-classified, heavy-particle fraction of CM that contained more fibre than the light-particle fraction or parent CM was also associated with reduced diet nutrient digestibility (Zhou et al., 2013). *B. juncea* contains less fibre, specifically cellulose and lignin than *B. napus*

(Bell and Shires, 1982; Bell et al., 1998). The lower fibre content due to thinner seed coat of *B. juncea* increased CATTD of DM and GE for *B. juncea* CM compared with for *B. napus* CM (Montoya and Leterme, 2009; Zhou et al., 2013).

Energy digestibility is a key factor for accurate prediction of energy values of diets and energy retention for pig growth (Noblet et al., 1994). Decreased GE digestibility coincided with reduced diet DE and calculated NE values, similar to results obtained by feeding weaned pigs up to 240 g solvent-extracted *B. juncea* CM/kg (Landero et al., 2013). Diets had been formulated to equal NE value and SID Lys/MJ NE to meet nutrient requirements of young pigs from 10 to 20 kg (NRC, 1998). Diet formulation based on NE and SID amino acid systems reduces risks associated with feeding high-fibre, high-protein co-products (Zijlstra and Beltranena, 2013). Diet NE values were balanced by supplementing canola oil assuming a value of 22.46 MJ NE/kg (NRC, 1998). After the study, diet NE values were recalculated using the new NE value of 31.63 MJ/kg for canola oil (NRC, 2012). Thus, instead of constant NE value among diets, corrected oil NE value (MJ/kg) decreased calculated dietary NE from 10.55 to 10.30 MJ/kg in phase 1 and from 9.92 to 9.71 MJ/kg in Phase 2 for pigs fed 0 and 240 g extruded *B. juncea* expeller/kg, respectively. Thus, the unequal diet NE values might explain the observed ADFI and ADG responses.

Increasing inclusion of extruded *B. juncea* expeller increased growth performance of weaned pigs in the present study. The ADFI was greater for pigs fed increasing dietary extruded *B. juncea* expeller consistent with the paradigm that pigs fed diets lower in energy consume more feed to meet their energy requirement (Nyachoti et al., 2004). However, ADFI increased quadratically. We think that this increase was not linear because greater glucosinolate intake at high inclusion of extruded *B. juncea* expeller may have deterred a continued linear increase in

ADFI up to 240 g extruded *B. juncea* expeller/kg. Consequently, increasing extruded *B. juncea* expeller inclusion increased ADG quadratically. To relate ADG to energy intake, overall NE intake was calculated and was not constant, but increased quadratically with increasing extruded *B. juncea* expeller inclusion. Increased NE intake then caused increased growth rate of pigs that were in their energy-dependent phase of growth (Campbell and Dunkin, 1983).

In the present study, the SID Lys/MJ of NE increased with increasing extruded *B. juncea* expeller inclusion due to reducing diet NE value but constant SID Lys content. This increasing ratio of SID Lys/MJ to NE partly explained the increased ADG (Schneider et al., 2010). Pigs fed the control diet achieved the lowest NE intake and lowest SID Lys/MJ of NE, thereby reducing ADG and G:F. Previously, pigs fed increasing Lys:energy ratio and energy value increased their ADG (De La Llata et al., 2007). The interaction between Lys:energy ratio and dietary energy value influences growth performance (Smith et al., 1999, Schneider et al., 2010).

Dietary fibre content may also affect growth performance of pigs. Increasing fibre content in pig diets decreased protein and energy digestibility and thereby reduced growth performance of pigs in some studies (Freire et al., 1998; Dégen et al., 2007). In contrast, high dietary fibre content from corn distillers dried grains with solubles, soybean hulls, citrus pulp or wheat bran combined with sugar beet pulp did not affect ADFI, ADG and G:F of weaned pigs fed isoenergetic diets (Weber et al., 2008; Hermes et al., 2010). Thus, if nutrient profile of diets is maintained, weaned pigs are able to maintain growth performance when fed diets containing fibrous feedstuffs ranging in fibre fermentation and viscosity characteristics.

Glucosinolates are important anti-nutritional factors in CM with a bitter taste that may reduce feed intake and growth in pigs (Newkirk, 2009). Whether glucosinolates can explain the differential responses of weaned pigs fed high dietary inclusion of CM remains unclear. While

weaned pigs fed 200 g solvent-extracted or expeller-pressed *B. napus* CM/kg did not have reduced ADFI or ADG (Landero et al, 2011, 2012), pigs fed 240 g solvent-extracted *B. juncea* CM/kg had reduced ADFI and ADG early in the growth phase (Landero et al., 2013) and pigs fed 240 g extruded *B. juncea* expeller/kg had quadratically increased ADFI and ADG in the present study. High dietary content of glucosinolates induces liver and thyroid enlargement, iodine deficiency and imbalance of serum thyroid hormones in pigs (Schone et al., 1997; Tripathi and Mishra, 2007). Total glucosinolate content of extruded *B. juncea* expeller in the present study was similar to that of solvent-extracted *B. juncea* CM (Landero et al., 2013) and 2-fold greater than solvent-extracted *B. napus* CM (Landero et al., 2011). Inclusion of 240 g extruded *B. juncea* expeller/kg in the present study resulted in 2.6 μmol glucosinolate/g in diets, slightly greater than the maximum 2.5 μmol glucosinolates/g recommended for pig diets (Bell, 1993). Such high inclusion may have deterred sustained linear increases in ADFI for pigs fed 240 g extruded *B. juncea* expeller. Type of glucosinolate and its degradation products exert differential effects. The major glucosinolate in *B. juncea* is gluconapin and in *B. napus* is progoitrin (2-hydroxy-3-butenyl; Tripathi and Mishra, 2007; Slominski et al., 2012). Gluconapin tastes bitter with greater intensity than progoitrin (Fenwick et al., 1983). Gluconapin content was negatively correlated with growth performance and CP digestibility (Landero et al., 2013) in weaned pigs.

Apart from programs to breed low-glucosinolate canola (Love et al., 1990), heat during oil extraction or meal processing can degrade glucosinolates; thus, reducing glucosinolates and its breakdown products in CM (Schone et al., 1997; Tripathi and Mishra, 2007). Added moisture and heat reduced glucosinolates and their breakdown products in rapeseed press cake (Schone et al., 1997). Glucosinolate reduction varies among types of glucosinolate and processing conditions (Spragg and Mailer, 2007; Tripathi and Mishra, 2007). Glucosinolates were reduced

by 50% by expeller pressing and by 80% with solvent extraction that occurred mostly during desolventising and toasting (Spragg and Mailer, 2007) resulting in less glucosinolates in solvent-extracted than expeller-pressed CM (Seneviratne et al., 2011b) and solvent-extracted versus expeller-pressed *B. napus* CM (Landero et al., 2011, 2012). However, total glucosinolate content of extruded *B. juncea* expeller in the present study was similar to that of solvent-extracted *B. juncea* CM (Landero et al., 2013). Effect of extrusion prior to pressing on glucosinolate reduction requires clarification to increase the feed quality and acceptance of CM.

3.6. Conclusion

Increasing extruded *B. juncea* expeller inclusion linearly decreased diet nutrient digestibility and energy values and quadratically increased overall ADFI and ADG, but did not affect overall G:F in weaned pigs. The lack of a linear increase in feed intake up to the greatest inclusion (240 g/kg) was attributed to the bitter glucosinolate content (gluconapin, 88% of total glucosinolates) of extruded *B. juncea* expeller.

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Table 3.1. Ingredient composition and analysed nutrient content (g/kg diet as fed) of the experimental diets

	Extruded *Brassica juncea* expeller (g/kg diet)									
	Phase 1 diets					Phase 2 diets				
	0	60	120	180	240	0	60	120	180	240
Ingredient										
Wheat, ground	542.1	547.0	552.0	556.9	564.0	665.2	671.0	676.3	681.9	687.4
Soybean meal, 460 g CP/kg	240.0	180.0	120.0	60.0	-	240.0	180.0	120.0	60.0	-
Extruded *Brassica juncea* expeller[a]	-	60.0	120.0	180.0	240.0	-	60.0	120.0	180.0	240.0
Lactose	50.0	50.0	50.0	50.0	50.0	-	-	-	-	-
Canola oil	55.0	49.0	43.0	37.0	29.0	26.0	19.5	13.0	6.5	-
Herring fish meal, 700 g CP/kg	40.0	40.0	40.0	40.0	40.0	25.0	25.0	25.0	25.0	25.0
Soy protein concentrate, 560 g CP/kg	30.0	30.0	30.0	30.0	30.0	-	-	-	-	-
Limestone	9.7	9.5	9.3	9.1	8.9	10.6	10.3	10.0	9.7	9.4
Mono/di-calcium phosphate	9.0	8.5	8.0	7.5	7.0	9.6	9.2	8.7	8.3	7.8
Celite[b]	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Vitamin premix[c]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Mineral premix[d]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Salt	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
L-Lysine HCl, 780 g/kg	0.3	1.5	2.6	3.8	5.0	0.3	1.5	2.7	3.8	5.0
L-Threonine, 990 g/kg	0.4	0.8	1.2	1.6	2.0	-	4.0	8.0	1.2	1.6
DL-Methionine, 990 g/kg	0.3	0.4	0.4	0.5	0.5	-	0.1	0.1	0.1	0.1
L-Tryptophan, 990 g/kg	-	0.1	0.2	0.3	0.4	-	0.1	0.2	0.3	0.4
Choline chloride, 600 g/kg	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Analysed nutrients[e]										
Moisture	107.2	110.2	112.6	99.6	98.4	98.7	118.3	108.8	107.4	103.8
Crude protein	228.7	221.1	218.7	214.5	212.6	233.7	213.5	223.2	206.6	198.5

Ether extract	68.3	70.1	70.1	79.2	79.5	36.2	39.1	42.0	45.7	45.6
Crude fibre	18.1	19.1	20.7	21.9	23.0	24.1	23.7	25.2	25.9	27.2
Ash	61.4	61.6	59.4	60.1	58.8	62.4	58.3	58.1	59.4	56.7
Acid detergent fibre	37.7	40.1	44.3	48.3	52.3	38.9	40.8	47.0	49.4	55.9
Neutral detergent fibre	84.7	88.0	93.1	98.1	108.3	110.8	132.8	135.1	143.1	144.7
Starch	314.8	317.4	330.1	318.0	323.3	418.0	427.1	414.1	410.4	431.0
Gross energy (MJ/kg)	17.46	17.39	17.52	17.50	17.64	16.96	16.58	16.87	16.75	16.99

[a] Canola seed was sourced commercially from southern Saskatchewan, Canada and then extruded before expeller-pressing at Apex Nutri-Solutions Inc. (Edberg, Alberta, Canada).

[b] Celite 281 (World Minerals Inc., Santa Barbara, CA, USA) used as acid insoluble ash.

[c] Supplied per kilogram of diet: 7500 IU of vitamin A, 750 IU of vitamin D, 50 IU of vitamin E, 37.5 mg of niacin, 15 mg of pantothenic acid, 2.5 mg of folacin, 5 mg of riboflavin, 1.5 mg of pyridoxine, 2.5 mg of thiamine, 2000 mg of choline, 4 mg of vitamin K, 0.25 mg of biotin and 0.02 mg of vitamin B_{12}.

[d] Supplied per kilogram of diet: 125 mg of Zn, 50 mg of Cu, 75 mg of Fe, 25 mg of Mn, 0.5 mg of I and 0.3 mg of Se.

[e] Phase 1 and 2 diets were formulated to contain (as fed): 10.01 and 9.67 MJ NE/kg, 4.90 and 4.43 g SID lysine/Mcal NE, respectively.

Table 3.2. Nutrient content (g/kg, as is) of extruded *Brassica (B.) juncea* expeller

Item	Extruded *B. juncea* expeller
Moisture	49.8
Crude protein	343.7
Crude fat[a]	168.5
Crude fibre	59.9
Acid detergent fibre	126.7
Neutral detergent fibre	195.3
Total dietary fibre	257
Ash	63.4
Calcium	6.3
Phosphorus	20.7
Indispensable amino acids	
Arginine	20.7
Histidine	8.7
Isoleucine	13.9
Leucine	24.4
Lysine	17.2
Methionine	6.2
Phenylalanine	14.2
Threonine	13.6
Tryptophan	4.1
Valine	17.7
Total amino acids[b]	311.2
Available lysine	15.7
Total glucosinolates[c], μmol/g	10.89

[a] Extruded *B. juncea* expeller contained the following fatty acids (g/100 g total fatty acids): C16:0, 4.7; C16:1, 0.2; C18:0, 2.5; C18:1 n-9, 60.2; C18:2 n-6, 15.4; C18:3 n-3, 8.6; C20:0, 0.6; C20:1 n-9, 1.4; saturated fatty acids, 7.8; monounsaturated fatty acids, 61.8 and polyunsaturated fatty acids, 24.0.

[b] Extruded *B. juncea* expeller contained the following dispensable amino acids (g/kg): alanine, 14.6; aspartic acid, 23.6; cysteine, 7.0; glutamic acid, 59.2; glycine, 16.5; proline, 22.3; serine, 12.5 and tyrosine, 10.3.

[c] Extruded *B. juncea* expeller contained the following glucosinolates (μmol/g): allyl, 0.2; 3-butenyl, 9.66; 4-pentenyl, 0.39; 2-OH-3-butenyl, 0.83; phenylethyl, 0.19 and 4-OH-3-CH_3-indolyl, 1.69.

Table 3.3. Apparent total tract digestibility coefficients (CATTD) of nutrients and digestible energy (DE) and net energy (NE) values (as-fed) of Phase 1 and 2 diets resulting from feeding increasing levels of extruded *Brassica (B.) juncea* expeller in substitution for soybean meal to weaned pigs[a]

	Extruded *B. juncea* expeller, g/kg diet						*P*-value	
Variable	0	60	120	180	240	SEM[b]	Linear	Quadratic
CATTD								
Dry matter								
Phase 1 diets	0.855	0.846	0.836	0.827	0.817	0.003	<0.001	0.817
Phase 2 diets	0.851	0.845	0.837	0.827	0.818	0.002	<0.001	0.233
Crude protein								
Phase 1 diets	0.832	0.824	0.808	0.797	0.779	0.005	<0.001	0.360
Phase 2 diets	0.843	0.835	0.823	0.811	0.797	0.003	<0.001	0.384
Gross energy								
Phase 1 diets	0.867	0.862	0.853	0.844	0.834	0.003	<0.001	0.376
Phase 2 diets	0.859	0.860	0.852	0.843	0.832	0.002	<0.001	0.012
Energy value								
DE, MJ/kg								
Phase 1 diets	15.1	15.0	14.9	14.8	14.7	0.05	<0.001	0.788
Phase 2 diets	14.6	14.3	14.4	14.1	14.1	0.04	<0.001	0.057
NE, MJ/kg								
Phase 1 diets	10.7	10.6	10.6	10.5	10.5	0.04	<0.001	0.943
Phase 2 diets	10.3	10.1	10.1	10.1	10.1	0.03	<0.001	0.039

[a] The Phase 1 and 2 diets were fed from days 0 to 14 and 15 to 35, respectively. Least-squares means based on 12 pen observations of 4 pigs per diet.

[b] Standard error of the mean.

Table 3.4. Growth performance of weaned pigs fed Phase 1 and 2 diets with increasing inclusion of extruded *Brassica (B.) juncea* expeller in substitution for soybean meal[a]

	Extruded *B. juncea* expeller (g/kg diet)						*P*-value	
Variable	0	60	120	180	240	SEM[b]	Linear	Quadratic
ADFI[b] (g/d)								
Day 0-7	207	222	205	210	204	15	0.423	0.429
Day 8-14	468	513	505	505	492	22	0.469	0.097
Day 15-21	659	723	745	783	727	28	0.011	0.011
Day 22-28	1004	1056	1064	1019	1000	26	0.591	0.038
Day 29-35	1182	1282	1297	1235	1215	58	0.880	0.019
Day 0-35[c]	704	759	764	750	727	21	0.402	<0.001
ADG[b] (g/d)								
Day 0-7	193	211	202	202	208	20	0.513	0.699
Day 8-14	330	380	392	397	389	30	0.035	0.087
Day 15-21	411	475	497	543	501	16	<0.001	0.001
Day 22-28	686	695	693	668	675	21	0.418	0.698
Day 29-35	778	787	824	802	786	18	0.562	0.083
Day 0-35	479	510	522	522	516	13.18	0.005	0.003
Feed efficiency (G:F)								
Day 0-7	0.99	1.02	1.05	1.04	1.10	0.16	0.110	0.807
Day 8-14	0.69	0.73	0.77	0.78	0.78	0.032	0.001	0.171
Day 15-21	0.63	0.66	0.67	0.69	0.69	0.016	0.004	0.228
Day 22-28	0.68	0.66	0.65	0.66	0.68	0.011	0.595	0.008
Day 29-35	0.67	0.62	0.65	0.66	0.66	0.027	0.706	0.089
Day 0-35	0.72	0.74	0.75	0.77	0.79	0.041	0.140	0.978

[a] The Phase 1 and 2 diets were fed from days 0 to 14 and 15 to 35, respectively. Least-squares means based on 12 pen observations of 4 pigs per diet.

[b] SEM = Standard error of the mean; ADFI = average daily feed intake; ADG = average daily gain.

[c] Net energy intake (MJ/day; SEM=0.21; $P<0.01$, quadratically): 7.28, 7.77, 7.81, 7.61 and 7.41; Standardised ileal digestible lysine intake (g/day, SEM=0.20; $P<0.01$ linearly; $P<0.001$ quadratically): 6.92, 7.57, 7.70, 7.66 and 7.51; for pigs fed 0, 60, 120, 180 and 240 g/kg EP *B. juncea* CM, respectively.

CHAPTER 4 – Effects of feeding fermented wheat with *Lactobacillus reuteri* on gut morphology, intestinal fermentation, nutrient digestibility, and growth performance in weaned pigs

4.1. Abstract

Feeding fermented feed to weaned pigs may improve nutrient digestibility and gut health and thereby reduce diarrhea incidence. Effects of feeding wheat grain fermented for 24 h with *Lactobacillus reuteri* were evaluated with 36 weaned pigs (7.3 kg BW). Fermented wheat grain contained (DM basis) 14.2% CP, 0.45% chemically available Lys, and 7.8% NDF, whereas unfermented wheat grain contained 16.4% CP, 0.45% chemically available Lys, and 9.9% NDF. Pigs were fed 6 mash wheat-based diets balanced for water content during 2 phases: Phase 1 diets for 1 wk (d 0–7) with 20% unfermented or fermented wheat and, subsequently, Phase 2 diets for 2 wk (d 8–21) with 50% unfermented or fermented wheat. The 6 diets were unfermented wheat (CTRL), unfermented wheat and chemically acidified (ACD), fermented wheat with *L. reuteri* TMW1.656 and 10% sucrose, fermented wheat with *L. reuteri* TMW1.656 and 5% glucose + 5% fructose, fermented wheat with *L. reuteri* LTH5794 and 10% sucrose, and fermented wheat with *L. reuteri* LTH5794 and 5% glucose + 5% fructose. Diets were formulated to provide 2.5 and 2.4 Mcal NE/kg and 5.3 and 5.0 g standardized ileal digestible Lys/Mcal NE for Phase 1 and 2 diets, respectively. Feeding fermented wheat reduced ($P < 0.05$) apparent total tract digestibility (ATTD) of diet DM (84.7 vs. 85.4 %), GE (84.4 vs. 85.3 %), and CP (81.8 vs. 83.6 %) for d 15 through 21 compared with CTRL and ACD diets. Weaned pigs fed fermented wheat diets had lower ($P < 0.05$) ADFI than pigs fed CTRL and ACD diets for d 0 through 7. The ADFI, ADG, and G:F did not differ between pigs fed fermented and unfermented diets.

Concentrations of acetic, propionic, and branched-chain fatty acids and total VFA in feces increased ($P < 0.05$) for pigs fed fermented wheat diets containing exopolysaccharides (EPS). However, VFA did not differ in ileal digesta. Villus height in the duodenum and jejunum increased in pigs fed fermented wheat without EPS ($P < 0.05$) compared with pigs fed fermented wheat with EPS. However, pigs fed the CTRL and ACD diets had longer ($P < 0.05$) villi and deeper crypt in the ileum than pigs fed fermented wheat. The ratio of villus height to crypt depth among treatments did not differ in the 3 segments of small intestine of weaned pigs. In conclusion, feeding fermented wheat grain diets to weaned pigs did not affect gut morphology, intestinal fermentation, growth performance, and ATTD of nutrients; however, EPS stimulated hindgut fermentation and may promote health benefits.

Key words: exopolysaccharide, fermented wheat, growth performance, weaned pig

4.2. Introduction

Weaning imposes various stresses to piglets including consuming dry feed, change of environment, and challenges with pathogens. Combined with an immature digestive tract and immune system, these sudden changes increase susceptibility of piglets to post-weaning diarrhea (**PWD**; Lallès et al., 2007). Removal of antibiotics as growth promoters from swine feed may further increase diarrhea incidence (Stein, 2002; Laine et al., 2008). Although various antibiotic alternatives exist, combined approaches that promote health benefits, prevent enteric diseases, and manage growth of weaned pigs are more effective than single approaches (Stein and Kil, 2006). Fermentation of cereal grains may provide combined benefits to young pigs.

Lactobacillus reuteri TMW1.656 and LTH5794 produce the exopolysaccharides (**EPS**) reuteran and levan in the presence of sucrose as a substrate, respectively (Gänzle and Vogel, 2003; Gänzle et al., 2007). Levan and reuteran inhibit adhesion of enterotoxigenic *Escherichia coli* (ETEC) to porcine cells (Wang et al., 2010), and decrease net fluid loss induced by ETEC, indicating their potential to reduce PWD losses (Chen et al., 2014). Fermentation of wheat supports production of **EPS** by *L. reuteri* (Tieking and Gänzle, 2005); thus, feed fermentation may deliver EPS to weaned pigs. However, effects of feeding fermented wheat grain require evaluation in weaned pigs in terms of individual effects of fermentation, organic acids, and the EPS reuteran and levan.

Our null hypothesis was that pigs fed fermented wheat grain with *L. reuteri* TMW1.656 or LTH5794 with and without EPS would have similar gut morphology, intestinal fermentation, nutrient digestibility, and growth performance. The objectives were to determine effects of feeding diets containing unfermented or fermented wheat grain with or without EPS on apparent total tract digestibility of GE, CP, and DM of diets, growth performance, and intestinal morphology and fermentation products in weaned pigs.

4.3. Materials and methods

4.3.1. Animal housing and experimental design

Animal care use and procedures were reviewed by the University of Alberta Animal Care and Use Committee for Livestock, and followed principles established by the Canadian Council on Animal Care (2009). The study was performed at the Swine Research and Technology Centre (SRTC), University of Alberta (Edmonton, AB, Canada).

In total, 36 crossbred pigs (Duroc × Large White/Landrace F_1; Hypor, Regina, SK, Canada) were weaned at 19 ± 1 d of age. Pigs were selected based on BW (7.3 ± 1.7 kg) at weaning. Pigs were individually housed in metabolism pens.

Six dietary treatments were randomly allotted to pigs housed in adjacent pens by area of the room in a randomized block design for 6 replicate pens per treatment. Individual metabolism pens (0.5 m wide by 1.22 m long by 0.76 m high) were made of solid plastic planking, had a window allowing nose-to-nose contact with the neighbor piglet, and were raised 0.8 m from the concrete floor. Pen floors were fully slatted with extruded plastic flooring. Each pen was equipped with a stainless steel, wet/dry self-feeder (0.17 m wide by 0.15 m high trough) attached to the front of the pen, and a single cup drinker attached to the side wall (0.09 m above pen floor). Room temperature was maintained within the thermoneutral zone of pigs, using a negative pressure ventilation system. Lighting was provided for a 12-h light (0600–1800 h) and 12-h dark cycle. Pigs had free access to feed and water during the trial.

4.3.2. Experimental diets

Pigs were fed 6 mash wheat-based diets balanced for water content during growth phases: Phase 1 diets for 1 wk (d 0–7) with 20% unfermented or fermented wheat grain and, subsequently, Phase 2 diets for 2 wk (d 8–21) with 50% unfermented or fermented wheat grain. Six diets were prepared as described by Yang et al. (2015a): 1) unfermented wheat (**CTRL**), 2) unfermented wheat and chemically acidified (**ACD**), 3) fermented wheat with *L. reuteri* TMW1.656 and 10% sucrose, 4) fermented wheat with *L. reuteri* TMW1.656 and 5% glucose + 5% fructose, 5) fermented wheat with *L. reuteri* LTH5794 and 10% sucrose, and 6) fermented wheat with *L. reuteri* LTH5794 and 5% glucose + 5% fructose. *Lactobacillus reuteri* TMW1.656 is a sourdough isolate with the gene coding for reuteransucrase, *gtfA*; whereas *L. reuteri* LTH5794

originates from the human intestine with gene *ftfA* coding for levansucrase (Wang et al., 2010; Yang et al., 2015a). Diets without antimicrobials or growth promoters were formulated to provide 2.5 and 2.4 Mcal NE/kg and 5.3 and 5.0 g standardized ileal digestible Lys/Mcal NE for Phase 1 and 2 diets, respectively. Titanium dioxide was added as an indigestible marker to diets.

The initial wheat sourdough was prepared in the lab using ground wheat grain (Hard Red Spring), water at the ratio of 1:1 (wt/vol), 10% sucrose (wt/wt), and cell cultures of either *L. reuteri* TMW1.656 or *L. reuteri* LTH5794 with approximately 10^7 cfu/g. The mixture was incubated at 37°C for 24 h. Subsequently, the fermented wheat was used as the first inoculum for additional wheat fermentation batches at the SRTC. Additional batches of ground wheat with equal amount of water, 10% sucrose, and 10% initial sourdough were incubated at 37°C for 24 h. Only 10% of fermented wheat was used for backslopping the subsequent batches of wheat fermentation while the rest was incorporated in the diets. Cell counts and pH measurement were conducted daily for quality control of wheat grain fermentation. The seed sourdough was replaced by a new laboratory-grown cultures after 4 batches of backslopping the fermentation at SRTC.

The combination of 5% glucose and 5% fructose (wt/wt) was used in place of sucrose for unfermented wheat, chemically acidified wheat and fermented wheat without added sucrose. The chemically acidified and unfermented wheat were mixed before feeding. Four parts of lactic acid (80%) and one part of glacial acetic acid (100%) were included in the mixture of ground wheat, water, 5% glucose and 5% fructose (wt/wt) for the chemically acidified wheat. The unfermented wheat was simply made by combining ground wheat, water, 5% glucose and 5% fructose (wt/wt). Experimental diets were freshly prepared by mixing 20% unfermented or fermented

wheat with 80% feed base for Phase 1 diets and 50% unfermented or fermented wheat with 50% feed base for Phase 2 diets.

4.3.3. Sample and data collection

Feed added and leftovers were weighed daily at 0800 h. Individual pigs were weighed weekly to calculate ADFI, ADG, and feed efficiency (G:F). Freshly voided feces were collected from 0800 to 1600h by grab sampling from pen floor on Days 5 and 6, 12 and 13, and 19 and 20 and stored at –20^{o}C. Feces were then thawed, homogenized, sub-sampled, and freeze-dried.

Diarrheal incidence was assessed for each pig throughout the experiment. Fecal consistency was scored twice a day using a 5-point scoring system with 1 as firm (no diarrhea), 2 as soft, 3 as moist or solid-liquid, 4 as liquid (diarrhea), and 5 as watery/projectile diarrhea (severe diarrhea) as previously described (Madec et al., 2000).

At the end of the experiment on Day 20 and 21, pigs were euthanized using captive bolt stunning technique. The abdomen was immediately opened and the digestive tract was clamped and removed. Digesta from the stomach, duodenum, jejunum, terminal ileum, colon, and caecum was collected in sterile containers and frozen at –20^{o}C.

Two-centimeter segments of the duodenum, jejunum, and ileum were opened lengthwise, quickly rinsed in saline (0.9% NaCl) and fixed in scintillation tubes filled with 10% formalin. The tissues were eventually embedded in paraffin and sectioned. Slides were stained with hematoxylin and eosin. Villus height and crypt depth were measured using light microscopy with the AxioVision Imaging software (version 4.7.2; Carl Zeiss AG, Jena, Germany) and were averaged for 15 measurements from each intestinal segment.

4.3.4. Chemical analyses

Samples of freeze-dried unfermented and fermented wheat, diets, and feces were ground through a 1-mm screen in a centrifugal mill (model ZM200, Retsch GmbH, Haan, Germany). Unfermented and fermented wheat were analyzed for N (method 984.13A-D), ADF inclusive of residual ash (method 973.18**)**, ash (method 942.05), starch (assay kit STA-20; Sigma, St. Louis, MO), Ca (method 968.08), P (method 946.06), AA (method 982.30E), chemically available Lys (method 975.44) as described by AOAC (2006), and NDF (Holst, 1973). Diets were analyzed for DM (method 930.15, AOAC, 2006), GE using an adiabatic bomb calorimeter (model 5003; Ika-Werke GMBH & Co. KG, Staufen, Germany), CP (N × 6.25; method 988.05; AOAC, 2006), ether extract, starch, crude fiber, ADF, NDF, AA, chemically available Lys, and TiO_2 (Myers et al., 2004). Feces were analyzed for DM, GE, CP, and TiO_2.

Based on results of chemical analyses, the apparent total tract digestibility (**ATTD**) of DM, GE, and CP and DE values were calculated using the marker concentration of feces relative to feed by the indicator method (Adeola, 2001). The NE value of diets was calculated based on DE value and chemical composition (CP, ether extract, starch, and ADF) using equation [5] of Noblet et al. (1994) and adopted by the NRC (2012). Volatile fatty acids were identified and measured using gas chromatography (model 3400; Varian, Walnut Creek, CA) with isocaproic acid as an internal standard.

4.3.5. Statistical analyses

Data were analyzed using the MIXED procedure of SAS (version 9.2, SAS Inst. Inc., Cary, NC, USA) with pig as the experimental unit. The fixed effect of diet and the random effect of block were included in the statistical model. Growth performance data were analyzed as repeated measures with week as the repeated term. Initial BW was included as a covariate to analyze

growth performance. The effects of acid, fermentation, EPS, and fermentation with or without EPS were tested using single-degree-of-freedom contrasts. To test the hypotheses, $P < 0.05$ was considered significant.

4.4. Results

4.4.1. Chemical composition of fermented and unfermented wheat grain

With 10% addition of sucrose, fermented wheat after 24 h of fermentation contained 5.6 ± 1.0 g/kg reuteran and 3.2 ± 0.6 g/kg levan. Furthermore, fermented wheat contained other metabolites such as 80.7 ± 4.6 to 87.1 ± 5.8 mmol/kg lactate, 51.0 ± 4.2 to 57.3 ± 3.7 mmol/kg acetate, and 27.3 ± 4.9 to 36.2 ± 1.8 mmol/kg ethanol (Yang et al., 2015a). Fermented wheat grain contained 2% less CP on DM basis than unfermented wheat, whereas chemically-available Lys did not differ (Table 2). The Arg content was 0.18% lower whereas ornithine content was 0.1 % greater in fermented wheat than in unfermented wheat. Crude fiber, ADF, and NDF were 0.5, 1.2, and 2.1% lower, respectively, in fermented wheat than in unfermented wheat.

4.4.2. Diet digestibility

Feeding fermented wheat to weaned pigs reduced ($P < 0.05$; Table 3) ATTD of diet DM, GE, and CP by 0.7, 1.8, and 0.9 %, respectively, during d 15 through 21 compared with feeding CTRL and ACD diets. Feeding fermented wheat did not affect diet DE and calculated NE values.

4.4.3. Growth performance of weaned pigs

Diarrhea was not observed during the experiment (data not shown). Weaned pigs fed fermented wheat grain diets had 17% lower ADFI ($P < 0.05$; Table 4) than pigs fed CTRL and ACD diets during d 0 through 7, but did not differ during d 8 through 21. Pigs fed fermented wheat diets tended to have lower ($P < 0.10$) ADG and G:F than pigs fed CTRL and ACD during d 15

through 21. However, ADFI, ADG, and G:F did not differ between pigs fed unfermented and fermented wheat diets for the entire study.

4.4.4. Histomorphology of the small intestine

Villi in the duodenum and jejunum were 11% longer ($P < 0.05$; Table 5) in pigs fed fermented wheat without EPS compared with pigs fed fermented wheat with EPS, whereas crypts were 9% deeper in the duodenum ($P < 0.05$). Villus height and crypt depth in the duodenum and jejunum did not differ between pigs fed fermented and unfermented diets. However, pigs fed unfermented wheat had 11% longer villi and 16% deeper crypts in the ileum than pigs fed fermented wheat diets ($P < 0.05$). The ratio of villus height to crypt depth did not differ in the small intestine of weaned pigs.

4.4.5. VFAs in ileal digesta and feces

Ileal digesta VFA did not differ among dietary treatments (Table 6). The concentrations of acetic, propionic, and branched-chain fatty acids and total VFA in feces increased by 33, 17, 30, and 27 %, respectively ($P < 0.05$; Table 7), in pigs fed fermented wheat grain with EPS compared with those fed fermented diets without EPS. Fecal VFA concentrations did not differ between pigs fed unfermented and fermented wheat diets. The concentration of butyric acid in feces was not affected by dietary treatments.

4.5. Discussion

4.5.1. Advantages of fermentation of carbohydrate-rich ingredients in swine feeding

Feed fermentation is considered in swine production as alternative for feed antibiotics (Stein and Kil, 2006; Canibe and Jensen, 2012). Fermentation of carbohydrate-rich ingredients is preferred to fermentation of complete feed. The consistent composition and low buffering capacity of

cereals rapidly reduces pH and subsequently stabilizes pH (Missotten et al., 2010). Additionally, fermentation of grain increases nutrient availability by activation of a variety of intrinsic cereal enzymes, leading to degradation of fiber and anti-nutritional factors in cereals, particularly phytate. Furthermore, fermentation of grain prevents the loss of synthetic AA and protein degradation if complete feed is fermented (Moran et al., 2006; Missotten et al., 2010).

4.5.2. Chemical changes in fermented wheat grain with *Lactobacillus reuteri*

Lactobacillus reuteri is a symbiont of pigs that forms stable populations in the pars oesophagus and also occurs in cereal fermentations (Walter, 2008; Su et al., 2012). Most strains of *L. reuteri* produce EPS from sucrose. The EPS formation supports biofilm formation by *L. reuteri* and is needed for colonization of the upper intestinal tract of mice (Walter et al., 2008). Feed fermentation with *L. reuteri* changed nutrient content of wheat. During fermentation, the metabolic activities of *L. reuteri* and activity of cereal enzymes degrade carbohydrates, proteins, and other phenolic compounds in wheat grain. These breakdown products in turn serve as substrates for bacterial growth (Gänzle, 2014). In the present study, fermented wheat grain contained less CP, crude fiber, ADF, and NDF than unfermented wheat. Starch and fat content marginally varied.

Although total AA in fermented wheat grain was reduced, the content of Lys and available Lys was not affected. This finding is similar to observations that fermentation of wheat by lactobacilli converts Gln, Glu and Arg but not Lys (Gänzle, 2014). The lower content of Arg and greater content of ornithine in fermented wheat resulted from the conversion of Arg into ornithine by *L. reuteri* via the arginine-deiminase pathway (Gänzle et al., 2007).

4.5.3. Digestibility of nutrients in fermented feed

Wheat fermentation with *L. reuteri* offers several features similar to feed digestion in the stomach of pigs with the presence of lactic acid, organic acids, enzymes, and commensal bacteria *L. reuteri*. Feeding fermented wheat grain contributes to digestion by modifying raw feedstuffs before feeding, maintaining the low gastric pH, which is important to weaned pigs for protection against coliforms and denaturation of protein, especially when secretion of HCl in the stomach is limited after weaning (Brooks et al., 2001; Yen, 2001). Therefore, fermentation of cereal grains increased nutrient digestibility in previous studies (Shekib, 1994; Cho et al., 2013).

Despite these advantages of fermentation, feeding fermented wheat to weaned pigs did not increase nutrient digestibility in the present study. Similar to our results, fermented feed did not increase total tract digestibility of nutrients in growing pigs (Pedersen and Stein, 2010). Digestibility of nutrients can be affected by voluntary feed intake of pigs. The presence of feed in the digestive tract stimulates the secretion of pancreatic enzymes and thus helps increase digestibility (Makkink et al., 1994).

4.5.4. Effects of fermented feed on growth performance

Pigs fed fermented wheat grain in the present study had lower feed intake in the first week compared with pigs fed unfermented wheat. However, wheat fermentation did not affect ADFI, ADG, and G:F of pigs for the entire trial similar to other studies. Pigs fed fermented liquid feed ate less and gained less weight than pigs fed unfermented liquid feed (Canibe and Jensen, 2003).

Low feed intake can be associated with palatability, which is influenced by pH, concentration of organic acids produced during fermentation, and the flavor and texture of the feed. High levels of acetic acid, butyric acid, and biogenic amines that resulted from spontaneous fermentation reduced palatability of pig feed (Beal et al., 2005; Canibe et al., 2010; Missotten et

al., 2010). In the present study, production of reuteran and levan from sucrose by *L. reuteri* TMW1.656 and LTH5794, respectively, reduced sucrose and increased acetate in fermented wheat (Korakli et al., 2001). Thus, the sweet taste of fermented wheat grain consisting of reuteran or levan is reduced and makes the feed less palatable to young pigs. It explains the numerical decrease in feed intake of pigs fed fermented diets with reuteran or levan compared with those fed fermented diets without EPS.

4.5.5. Effects of fermentation on gut morphology

Feeding fermented wheat grain did not influence intestinal morphology in the duodenum and jejunum but decreased villus height and crypt depth in the ileum of weaned pigs. However, pigs fed fermented diets without EPS had longer villi and deeper crypt than pigs fed fermented wheat with EPS in the duodenum, most likely due to greater feed intake. In line with our results, inclusion of fermented wheat in liquid diets increased villus height and the villus height–to–crypt depth ratio in the proximal small intestine of pigs during the first week after weaning (Scholten et al., 2002).

The greater increase in villus height and crypt depth in the proximal small intestine than in the distal small intestine may be related to the amount of available nutrients in the lumen (Vente-Spreeuwenberg et al., 2003). The presence of feed in the intestinal lumen of pigs has potential to stimulate cell proliferation, differentiation, and turnover, which then affects structure and functions of the intestinal epithelial layer. Hence, reduced feed intake at weaning is a major cause for villous atrophy in weaned pigs, because of not only the absence of feed in the gut but also energy deprivation (Pluske et al., 1997).

4.5.6. Effects of fermentation on gut microbiota and VFA concentrations

Fecal samples were used to characterize microbiota and measure VFA concentrations, because luminal microbial composition and fermentation in the colon was reflected in feces as turnover of colonic content (Eckburg et al., 2005; Gerritsen et al., 2011; Walker et al., 2011). The composition of fecal microbiota of weaned pigs, as described by Yang et al. (2015b), changed over time but 2 phyla, *Bacteroidetes* and *Firmicutes*, remained dominant throughout the study. Microbial diversity increased after weaning mostly attributable to the increased diversity of *Firmicutes* bacteria.

In the ileum, concentrations of individual and total VFA did not differ among dietary treatments, indicating lack of fermentation of EPS in the distal small intestine. This observation is similar to findings that fermentation of nondigestible oligosaccharides occurs in the large intestine, where bacterial diversity is greater than that in the ileum (Montagne et al., 2003; Niba et al., 2009).

The high proportion of acetate, followed by propionate, in fecal VFA is associated with the prevalence of bacteria in *Bacteroides-Prevotella* group that mainly produce acetate and propionate from carbohydrate fermentation (Louis et al., 2007). The increase in concentrations of VFA in feces of pigs fed fermented wheat with EPS, reuteran or levan, compared with those in pigs fed fermented wheat without EPS indicates the effect of EPS on microbial fermentation in the hindgut. Because reuteran and levan are not digested in the small intestine, they become fermentable substrates for proliferation of *Bacteroides* in the distal gut, especially *Bacteroides thetaiotaomicron* (Sonnenburg et al., 2010). *Bacteroides thetaiotaomicron* fully metabolize levan and partially degrade reuteran by enzymes and transport systems that are activated by upregulation of polysaccharide utilization loci specific for β-fructans and α-glucans utilization, in

response to levan and reuteran in the environment, respectively (Sonnenburg et al., 2010; van Bueren et al., 2015). Therefore, the present study demonstrated prebiotic effects of reuteran and levan in fermented wheat, as these EPS favored the growth of commensal flora *B. thetaiotaomicron* that increased VFA production in the hindgut and potentially benefits weaned pigs.

4.6. Conclusion

In conclusion, feeding fermented wheat grain with *L. reuteri* and reuteran or levan demonstrated the prebiotic effects of these exopolysaccharides on increased short-chain fatty acid production in the large intestine of weaned pigs. Fermented wheat without reuteran or levan increased villus height and crypt depth in the proximal small intestine over fermented wheat with exopolysaccharides, most likely related to feed intake. Digestibility of nutrients and growth performance of weaned pigs fed fermented wheat were not affected. Therefore, feeding fermented wheat with reuteran- or levan-producing *L. reuteri* may improve gut health of weaned pigs.

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Table 4.1. Ingredient composition (as-fed basis) and analyzed nutrient content (DM basis) of Phase 1 and Phase 2 diets

	Phase 1 diets[1]						Phase 2 diets					
			L. reuteri R[2]		*L. reuteri* L[3]				*L. reuteri* R		*L. reuteri* L	
Ingredient, %	CTRL	ACD	REU+	REU-	LEV+	LEV-	CTRL	ACD	REU+	REU-	LEV+	LEV-
Wheat, ground	20	20	-	-	-	-	50	50	-	-	-	-
Wheat, ground, fermented	-	-	20	20	20	20	-	-	50	50	50	50
Corn, ground	31.54	31.54	31.54	31.54	31.54	31.54	1.76	1.76	1.76	1.76	1.76	1.76
Soybean meal	15	15	15	15	15	15	15	15	15	15	15	15
Lactose	15	15	15	15	15	15	10	10	10	10	10	10
Canola meal	-	-	-	-	-	-	5	5	5	5	5	5
Wheat DDGS[4]	-	-	-	-	-	-	5	5	5	5	5	5
Soy protein concentrate	3	3	3	3	3	3	2.5	2.5	2.5	2.5	2.5	2.5
Herring fish meal	6	6	6	6	6	6	2.5	2.5	2.5	2.5	2.5	2.5
Canola oil	4	4	4	4	4	4	3.4	3.4	3.4	3.4	3.4	3.4
Limestone	1.15	1.15	1.15	1.15	1.15	1.15	1.1	1.1	1.1	1.1	1.1	1.1
Mono/di-calcium phosphate	1.3	1.3	1.3	1.3	1.3	1.3	1.0	1.0	1.0	1.0	1.0	1.0
Vitamin premix[5]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mineral premix[6]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Salt	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
TiO_2[7]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
L-Lys HCl	0.45	0.45	0.45	0.45	0.45	0.45	0.4	0.4	0.4	0.4	0.4	0.4
L-Thr	0.29	0.29	0.29	0.29	0.29	0.29	0.18	0.18	0.18	0.18	0.18	0.18
DL-Met	0.14	0.14	0.14	0.14	0.14	0.14	0.11	0.11	0.11	0.11	0.11	0.11
L-Trp	0.08	0.08	0.08	0.08	0.08	0.08	0.02	0.02	0.02	0.02	0.02	0.02
Choline chloride	0.05	0.05	0.05	0.05	0.05	0.05	0.03	0.03	0.03	0.03	0.03	0.03
Analyzed nutrients[8]												
GE, Mcal/kg	4.5	4.5	4.5	4.5	4.5	4.5	4.6	4.6	4.6	4.6	4.6	4.6
CP, %	21.6	21.3	21.5	21.7	21.2	21.8	24.4	24.0	25.1	24.9	25.0	25.2
Crude fat, %	5.79	5.95	5.80	5.92	5.97	6.26	5.84	5.69	5.18	5.30	5.16	4.98
NDF, %	7.74	8.16	7.93	8.04	7.71	8.14	10.8	9.54	12.6	10.5	10.5	11.8
ADF, %	3.61	3.08	3.43	3.23	3.55	3.56	4.95	5.04	4.70	5.04	5.21	5.22

Crude fiber, %	2.17	2.02	2.11	2.20	2.23	2.32	2.89	2.91	3.15	3.12	3.10	3.31
Ash, %	7.17	7.23	7.20	7.17	7.35	7.15	6.56	6.67	6.68	6.87	6.65	6.59

[1]CTRL = unfermented wheat; ACD = unfermented and chemically acidified wheat; REU+ = fermented wheat with *L. reuteri* TMW1.656 and 10% sucrose; REU– = fermented wheat with *L. reuteri* TMW1.656 and 5% glucose + 5% fructose; LEV+ = fermented wheat with *L. reuteri* LTH5794 and 10% sucrose; LEV– = *L. reuteri* LTH5794 and 5% glucose + 5% fructose.

[2]*Lactobacillus reuteri* R = *L. reuteri* strain TMW1.656, which produces the exopolysaccharide (EPS) reuteran in presence of sucrose.

[2]*Lactobacillus reuteri* L = *L. reuteri* strain LTH5794, which produces the EPS levan in presence of sucrose.

[4]DDGS = distiller's dried grains with solubles.

[5]Supplied per kg of diet: 7500 IU of vitamin A, 750 IU of vitamin D, 50 IU of vitamin E, 37.5 mg of niacin, 15 mg of pantothenic acid, 2.5 mg of folacin, 5 mg of riboflavin, 1.5 mg of pyridoxine, 2.5 mg of thiamine, 2000 mg of choline, 4 mg of vitamin K, 0.25 mg of biotin, and 0.02 mg of vitamin B_{12}.

[6]Supplied per kilogram of diet: 125 mg of Zn, 50 mg of Cu, 75 mg of Fe, 25 mg of Mn, 0.5 mg of I, and 0.3 mg of Se.

[7]Sigma-Aldrich Co., St. Louis, MO; indigestible marker.

[8]Phase 1 and 2 diets were formulated to provide (as-fed basis): 2.5 and 2.4 Mcal NE/kg, 5.3 and 5.0 g standardized ileal digestible Lys/Mcal NE, respectively. Phase 1 and Phase 2 diets were fed from d 0 to 7 and 8 to 21, respectively.

Table 4.2. Analyzed nutrient content (%) of the ground wheat grain (DM basis)

			Lactobacillus reuteri R[3]		*L. reuteri* L[4]	
Wheat	CTRL[1]	ACD[2]	REU+	REU-	LEV+	LEV-
Starch	58.1	59.4	63.5	59.4	56.7	58.1
CP	17.0	15.7	14.3	14.3	14.4	13.9
Crude fat	1.30	1.88	1.31	1.73	1.32	1.21
NDF	11.7	8.11	7.79	8.05	7.75	7.53
ADF	4.48	3.06	2.55	2.66	2.72	2.50
Crude fiber	2.66	2.15	1.85	1.87	1.90	1.90
Ash	2.34	2.51	2.22	2.53	2.47	2.55
P	0.42	0.44	0.41	0.42	0.40	0.41
Ca	0.10	0.40	0.36	0.44	0.43	0.45
Ornithine	0.00	0.00	0.10	0.10	0.11	0.10
Indispensable AA						
Arg	0.76	0.74	0.59	0.58	0.56	0.54
His	0.39	0.37	0.34	0.34	0.34	0.34
Ile	0.61	0.56	0.54	0.55	0.54	0.52
Leu	1.20	1.09	1.03	1.01	1.01	0.99
Lys	0.48	0.44	0.48	0.46	0.46	0.46
Met	0.28	0.26	0.27	0.27	0.26	0.26
Phe	0.83	0.79	0.72	0.73	0.71	0.71
Thr	0.47	0.43	0.41	0.41	0.40	0.40
Trp	0.17	0.17	0.15	0.15	0.15	0.14
Val	0.73	0.66	0.65	0.64	0.64	0.63
Total AA	16.6	15.4	14.4	14.5	14.2	14.2
Available Lys	0.47	0.43	0.46	0.46	0.44	0.44

[1]CTRL = unfermented wheat.

[2]ACD = unfermented and chemically acidified wheat.

[3]*Lactobacillus reuteri* R = *L. reuteri* strain TMW1.656, which produces the exopolysaccharide (EPS) reuteran in presence of sucrose. REU+ = fermented wheat with *L. reuteri* TMW1.656 and 10% sucrose; REU– = fermented wheat with *L. reuteri* TMW1.656 and 5% glucose + 5% fructose.

[4]*Lactobacillus reuteri* L = *L. reuteri* strain LTH5794, which produces the EPS levan in presence of sucrose. LEV+ = fermented wheat with *L. reuteri* LTH5794 and 10% sucrose; LEV– = *L. reuteri* LTH5794 and 5% glucose + 5% fructose.

Table 4.3. Apparent total tract digestibility (ATTD) of nutrients, DE value, and calculated NE value (DM basis) of diets fed to weaned pigs[1]

	Diet treatments							*P*-value[4]				
			Lactobacillus reuteri R[2]		*L. reuteri* L[3]							
Variable	CTRL	ACD	REU+	REU-	LEV+	LEV-	SEM	Acid	Ferm.	Exopol	Ferm. with EPS	Ferm. without EPS
ATTD of DM, %												
d 0–7	84.0	82.8	83.4	82.0	81.1	82.1	2.24	0.589	0.381	0.879	0.473	0.428
d 8–14	83.4	83.1	82.9	83.7	84.4	84.8	0.93	0.757	0.283	0.346	0.614	0.171
d 15–21	85.7	85.1	85.3	84.1	84.7	84.6	0.54	0.282	0.033	0.110	0.277	0.009
ATTD of CP, %												
d 0–7	75.6	75.1	76.9	72.0	72.2	74.2	3.58	0.894	0.494	0.586	0.740	0.412
d 8–14	81.4	79.8	79.3	79.9	80.2	82.8	1.76	0.407	0.964	0.186	0.494	0.556
d 15–21	83.7	83.4	82.8	80.7	81.7	82.0	0.97	0.743	0.005	0.192	0.063	0.003
ATTD of GE, %												
d 0–7	81.3	79.0	80.3	78.8	77.7	78.5	2.96	0.435	0.473	0.862	0.574	0.501
d 8–14	82.6	81.7	81.8	82.9	83.2	84.0	1.15	0.450	0.247	0.245	0.638	0.123
d 15–21	85.7	84.8	85.0	84.0	84.1	84.3	0.60	0.113	0.021	0.333	0.115	0.014
Energy value, Mcal/kg												
DE	3.79	3.74	3.78	3.77	3.76	3.78	0.04	0.125	0.740	0.800	0.869	0.683
Calculated NE	2.67	2.63	2.63	2.63	2.63	2.62	0.03	0.177	0.139	0.711	0.262	0.147

[1]Phase 1 and Phase 2 diets were fed from d 0 to 7 and 8 to 21, respectively. Least-square means based on 6 pigs per dietary treatment. CTRL = unfermented wheat; ACD = unfermented and chemically acidified wheat; REU+ = fermented wheat with *L. reuteri* TMW1.656 and 10% sucrose; REU– = fermented wheat with *L. reuteri* TMW1.656 and 5% glucose + 5% fructose; LEV+ = fermented wheat with *L. reuteri* LTH5794 and 10% sucrose; LEV– = *L. reuteri* LTH5794 and 5% glucose + 5% fructose.

[2]*Lactobacillus reuteri* R = *L. reuteri* strain TMW1.656, which produces the exopolysaccharide (EPS) reuteran in presence of sucrose.

[2]*Lactobacillus reuteri* L = *L. reuteri* strain LTH5794, which produces the EPS levan in presence of sucrose.

[4]Acid = acid addition (CTRL vs. ACD); Ferm. = fermentation (CTRL and ACD vs. REU+, REU–, LEV+ and LEV–); Exopol = fermentation with or without the EPS reuteran or levan (REU+ and LEV+ vs. REU– and LEV–); Ferm. with EPS = fermentation with EPS (CTRL and ACD vs. REU+ and LEV+); Ferm. without EPS = fermentation without EPS (CTRL and ACD vs. REU– and LEV–).

Table 4.4. Growth performance of weaned pigs fed unfermented and fermented wheat grain with *Lactobacillus reuteri*[1]

	Diet treatments							*P*-value[4]				
			L. reuteri R[2]		*L. reuteri* L[3]							
Variable	CTRL	ACD	REU+	REU-	LEV+	LEV-	SEM	Acid	Ferm.	Exopol	Ferm. with EPS	Ferm. without EPS
ADFI, g												
d 0–7	154	172	133	153	132	126	32	0.335	0.024	0.625	0.028	0.081
d 8–14	326	302	247	279	257	261	44	0.642	0.097	0.616	0.092	0.226
d 15–21	535	510	512	511	499	496	51	0.664	0.601	0.960	0.668	0.633
d 0–21	338	328	297	314	296	294	39	0.797	0.182	0.789	0.197	0.303
ADG, g												
d 0–7	76	121	87	99	58	76	33	0.036	0.151	0.326	0.086	0.453
d 8–14	253	210	223	214	206	223	52	0.496	0.700	0.923	0.702	0.773
d 15–21	444	462	413	408	368	420	42	0.670	0.056	0.424	0.042	0.198
d 0–21	258	264	241	240	211	240	33	0.841	0.174	0.538	0.139	0.376
G:F												
d 0–7	0.48	0.69	0.64	0.61	0.43	0.61	0.11	0.061	0.865	0.364	0.540	0.747
d 8–14	0.67	0.66	0.85	0.74	0.78	0.81	0.13	0.931	0.137	0.653	0.130	0.276
d 15–21	0.84	0.93	0.81	0.79	0.75	0.86	0.09	0.245	0.066	0.350	0.042	0.248
d 0–21	0.67	0.76	0.77	0.72	0.66	0.76	0.05	0.083	0.680	0.498	0.986	0.487

[1]Phase 1 and Phase 2 diets were fed from d 0 to 7 and 8 to 21, respectively. Least square means based on 6 pigs per dietary treatment. CTRL = unfermented wheat; ACD = unfermented and chemically acidified wheat; REU+ = fermented wheat with *L. reuteri* TMW1.656 and 10% sucrose; REU– = fermented wheat with *L. reuteri* TMW1.656 and 5% glucose + 5% fructose; LEV+ = fermented wheat with *L. reuteri* LTH5794 and 10% sucrose; LEV– = *L. reuteri* LTH5794 and 5% glucose + 5% fructose.

[2]*Lactobacillus reuteri* R = *L. reuteri* strain TMW1.656, which produces the exopolysaccharide (EPS) reuteran in presence of sucrose.

[2]*Lactobacillus reuteri* L = *L. reuteri* strain LTH5794, which produces the EPS levan in presence of sucrose.

[4]Acid = acid addition (CTRL vs. ACD); Ferm. = fermentation (CTRL and ACD vs. REU+, REU–, LEV+ and LEV–); Exopol = fermentation with or without the EPS reuteran or levan (REU+ and LEV+ vs. REU– and LEV–); Ferm. with EPS = fermentation with EPS (CTRL and ACD vs. REU+ and LEV+); Ferm. without EPS = fermentation without EPS (CTRL and ACD vs. REU– and LEV–).

Table 4.5. Villus height, crypt depth (µm), and the ratio of villus height to crypt depth (V:C) in the duodenum, jejunum, and ileum of weaned pigs fed unfermented and fermented wheat-based diets[1]

	Diet treatments							*P*-value[4]				
			L. reuteri R[2]		*L. reuteri* L[3]							
Variable	CTRL	ACD	REU+	REU-	LEV+	LEV-	SEM	Acid	Ferm.	Exopol	Ferm. with EPS	Ferm. without EPS
Duodenum												
Villus height	485	483	490	541	449	528	42.7	0.957	0.511	0.042	0.625	0.111
Crypt depth	322	340	315	309	294	362	21.2	0.386	0.403	0.047	0.087	0.761
V:C	1.51	1.43	1.56	1.74	1.56	1.46	0.12	0.509	0.164	0.680	0.311	0.159
Jejunum												
Villus height	492	497	453	511	460	514	36.5	0.893	0.667	0.039	0.155	0.484
Crypt depth	231	237	243	233	234	260	12.0	0.612	0.277	0.353	0.628	0.163
V:C	2.15	2.10	1.89	2.20	1.97	1.99	0.15	0.748	0.233	0.126	0.076	0.790
Ileum												
Villus height	373	374	325	319	328	355	20.9	0.960	0.003	0.455	0.004	0.022
Crypt depth	212	227	184	181	180	193	24.5	0.557	0.027	0.749	0.037	0.072
V:C	1.80	1.71	1.83	1.77	1.84	1.87	0.15	0.563	0.472	0.909	0.496	0.570

[1]Small intestinal tissues were collected after pig euthanasia on Day 21, fixed in 10% formalin, embedded in paraffin, sectioned, stained in hematoxylin and eosin, and measured under microscopy. Least square means based on 15 villi measured/segment per pig per dietary treatment. CTRL = unfermented wheat; ACD = unfermented and chemically acidified wheat; REU+ = fermented wheat

with *L. reuteri* TMW1.656 and 10% sucrose; REU– = fermented wheat with *L. reuteri* TMW1.656 and 5% glucose + 5% fructose; LEV+ = fermented wheat with *L. reuteri* LTH5794 and 10% sucrose; LEV– = *L. reuteri* LTH5794 and 5% glucose + 5% fructose.

[2]*Lactobacillus reuteri* R = *L. reuteri* strain TMW1.656, which produces the exopolysaccharide (EPS) reuteran in presence of sucrose.

[2]*Lactobacillus reuteri* L = *L. reuteri* strain LTH5794, which produces the EPS levan in presence of sucrose.

[4]Acid = acid addition (CTRL vs. ACD); Ferm. = fermentation (CTRL and ACD vs. REU+, REU–, LEV+ and LEV–); Exopol = fermentation with or without the EPS reuteran or levan (REU+ and LEV+ vs. REU– and LEV–); Ferm. with EPS = fermentation with EPS (CTRL and ACD vs. REU+ and LEV+); Ferm. without EPS = fermentation without EPS (CTRL and ACD vs. REU– and LEV–).

Table 4.6. Concentrations and molar ratios of acetate, propionate, butyrate, branched-chain fatty acids, and total VFA (µmol/g ileal digesta) of ileal digesta resulting from feeding unfermented and fermented wheat-based diets to weaned pigs[1]

	Diet treatments							*P*-value[4]				
			L. reuteri R[2]		*L. reuteri* L[3]							
Variable	CTRL	ACD	REU+	REU-	LEV+	LEV-	SEM	Acid	Ferm.	Exopol	Ferm. with EPS	Ferm. without EPS
Acetic acid	6.60	9.19	5.49	5.76	5.78	11.6	3.29	0.446	0.717	0.201	0.346	0.740
Propionic acid	0.17	0.68	0.44	0.31	0.41	0.41	0.26	0.067	0.843	0.721	0.996	0.724
Butyric acid	0.20	0.33	0.18	0.08	0.19	0.29	0.14	0.375	0.368	0.989	0.433	0.433
BCFA[5]	-	0.11	0.10	-	0.06	0.16	0.12	0.362	0.735	0.953	0.749	0.789
Total	7.09	10.4	6.44	6.24	6.65	12.6	3.72	0.394	0.741	0.285	0.418	0.802
Ratio (%:total VFA)												
Acetic acid	94.5	91.0	87.1	93.0	87.8	93.5	4.50	0.455	0.394	0.078	0.112	0.875
Propionic acid	1.87	5.22	6.57	4.29	5.87	3.35	2.54	0.204	0.355	0.188	0.154	0.879
Butyric acid	2.17	2.56	2.96	1.31	2.67	1.91	1.07	0.728	0.817	0.121	0.561	0.322
BCFA[5]	-	0.76	1.04	-	0.77	0.39	0.67	0.281	0.682	0.147	0.286	0.703
Acetic: Propionic	18.0	18.4	16.4	16.0	17.3	23.7	7.20	0.961	0.975	0.528	0.806	0.762
Acetic: Butyric	32.8	30.3	24.2	36.0	36.0	33.0	9.76	0.797	0.898	0.536	0.834	0.681

[1]Digesta was collected at the end of the experiment, on Day 21, after pig euthanasia. Least square means based on 6 replicate pigs per dietary treatment. CTRL = unfermented wheat; ACD = unfermented and chemically acidified wheat; REU+ = fermented wheat

with *L. reuteri* TMW1.656 and 10% sucrose; REU– = fermented wheat with *L. reuteri* TMW1.656 and 5% glucose + 5% fructose; LEV+ = fermented wheat with *L. reuteri* LTH5794 and 10% sucrose; LEV– = *L. reuteri* LTH5794 and 5% glucose + 5% fructose.

[2]*Lactobacillus reuteri* R = *L. reuteri* strain TMW1.656, which produces the exopolysaccharide (EPS) reuteran in presence of sucrose.

[2]*Lactobacillus reuteri* L = *L. reuteri* strain LTH5794, which produces the EPS levan in presence of sucrose.

[4]Acid = acid addition (CTRL vs. ACD); Ferm. = fermentation (CTRL and ACD vs. REU+, REU–, LEV+ and LEV–); Exopol = fermentation with or without the EPS reuteran or levan (REU+ and LEV+ vs. REU– and LEV–); Ferm. with EPS = fermentation with EPS (CTRL and ACD vs. REU+ and LEV+); Ferm. without EPS = fermentation without EPS (CTRL and ACD vs. REU– and LEV–).

[5]BCFA = branched-chain fatty acids, including isobutyrate, isovalerate, valerate, and caproic acid.

Table 4.7. Concentrations of acetate, propionate, butyrate, branched-chain fatty acids, and total VFA (µmol/g wet feces) of wet feces resulting from feeding unfermented and fermented wheat-based diets to weaned pigs[1]

	Diet treatments							*P*-value[4]				
			L. reuteri R[2]		*L. reuteri* L[3]							
Variable	CTRL	ACD	REU+	REU-	LEV+	LEV-	SEM	Acid	Ferm.	Exopol	Ferm. with EPS	Ferm. without EPS
Acetic												
d 0–7	53.4	77.4	78.3	60.1	72.8	38.4	19.8	0.222	0.806	0.075	0.469	0.256
d 8–14	39.8	48.7	45.0	29.8	62.3	35.3	10.4	0.401	0.857	0.008	0.213	0.124
d 15–21	36.5	31.3	49.6	30.4	31.9	33.0	7.08	0.469	0.597	0.081	0.183	0.664
d 0–21	43.2	52.4	57.6	40.1	55.8	36.4	7.72	0.231	0.941	0.001	0.106	0.082
Propionic												
d 0–7	16.2	23.7	21.0	19.2	21.9	14.2	3.78	0.051	0.698	0.092	0.578	0.228
d 8–14	13.2	14.3	13.5	11.2	16.4	13.7	2.21	0.635	0.980	0.122	0.443	0.418
d 15–21	13.5	12.8	15.3	12.0	11.8	12.6	1.53	0.664	0.808	0.258	0.718	0.436
d 0–21	14.3	16.9	16.6	14.1	16.7	13.7	1.56	0.093	0.723	0.016	0.352	0.125
Butyric												
d 0–7	5.79	6.40	6.53	6.77	7.12	4.92	1.42	0.658	0.780	0.347	0.470	0.806
d 8–14	4.88	4.94	5.29	5.34	4.88	4.58	1.07	0.955	0.861	0.869	0.815	0.945
d 15–21	6.48	5.80	5.31	5.14	5.04	5.60	1.02	0.510	0.177	0.792	0.194	0.295
d 0–21	5.71	5.71	5.71	5.75	5.63	5.08	0.66	0.998	0.673	0.585	0.928	0.523
BCFA[5]												
d 0–7	6.82	12.4	11.2	7.96	10.7	5.66	3.28	0.089	0.716	0.089	0.562	0.233
d 8–14	13.7	20.9	20.3	10.7	24.1	19.7	6.41	0.277	0.722	0.134	0.288	0.647
d 15–21	10.3	9.40	16.1	8.79	8.70	10.8	3.33	0.795	0.538	0.278	0.284	0.990
d 0–21	10.3	14.2	15.9	9.16	14.8	12.3	2.93	0.177	0.658	0.028	0.135	0.461
Total												
d 0–7	86.9	124.2	120.4	99.3	117.3	67.4	24.5	0.128	0.765	0.055	0.446	0.208
d 8–14	72.6	90.5	85.2	58.6	109.3	74.8	17.1	0.303	0.969	0.018	0.206	0.229
d 15–21	68.6	60.9	88.0	57.9	58.7	63.4	11.2	0.501	0.746	0.120	0.288	0.609
d 0–21	76.0	91.9	97.9	71.9	95.5	69.9	10.9	0.145	0.981	0.001	0.100	0.093

[1]The Phase 1 and Phase 2 diets were fed from Days 0 to 7 and Days 8 to 21, respectively. Least square means based on 6 pigs per dietary treatment. CTRL = unfermented wheat; ACD = unfermented and chemically acidified wheat; REU+ = fermented wheat with *L. reuteri* TMW1.656 and 10% sucrose; REU– = fermented wheat with *L. reuteri* TMW1.656 and 5% glucose + 5% fructose; LEV+ = fermented wheat with *L. reuteri* LTH5794 and 10% sucrose; LEV– = *L. reuteri* LTH5794 and 5% glucose + 5% fructose.

[2]*Lactobacillus reuteri* R = *L. reuteri* strain TMW1.656, which produces the exopolysaccharide (EPS) reuteran in presence of sucrose.

[2]*Lactobacillus reuteri* L = *L. reuteri* strain LTH5794, which produces the EPS levan in presence of sucrose.

[4]Acid = acid addition (CTRL vs. ACD); Ferm. = fermentation (CTRL and ACD vs. REU+, REU–, LEV+ and LEV–); Exopol = fermentation with or without the EPS reuteran or levan (REU+ and LEV+ vs. REU– and LEV–); Ferm. with EPS = fermentation with EPS (CTRL and ACD vs. REU+ and LEV+); Ferm. without EPS = fermentation without EPS (CTRL and ACD vs. REU– and LEV–).

[5]BCFA = branched-chain fatty acids, including isobutyrate, isovalerate, valerate, and caproic acid.

CHAPTER 5 – General Discussion

The general objectives of this thesis were to find dietary interventions that utilized canola co-products without affecting growth performance and to establish effect of feeding fermented wheat on intestinal microbiota to benefit health and growth performance of weaned pigs. Effects of different feeding strategies to weaned pigs were investigated such as feeding canola co-products and fermented wheat on digestibility of nutrients and growth performance. It was focused on the fiber content of canola meal and processing conditions to improve the nutritional value of canola meal and the whole diet. In this General Discussion, results from experiments are discussed in the context of practical perspectives.

The swine industry is facing challenges to raise healthy pigs without in-feed antibiotics, achieving optimal performance at a competitive cost and minimize environment impact (Stein, 2002; Aarnink and Verstegen, 2007). While enteric diseases are common in pig production, especially around weaning, feeding strategies play a key role for sustainable swine production in order to cope with recurring increases in feed cost and removal of in-feed antibiotics as growth promoters (de Lange et al., 2010). In addition, increased use of co-products in swine diets as alternatives to traditional feedstuffs provides opportunities to take advantages of local feedstuffs and thereby reduce feed cost hopefully without affecting pork quality (Woyengo et al., 2014). Apart from extrusion, fermentation was also applied to enhance the nutritional value of the diets, enhance gut health and thus increase pig growth performance, particularly after weaning (Canibe et al., 2007).

5.1. *B. juncea* canola meal

The experimental design and diet formulation in Chapter 2 was unique compared with other studies by solely including test diets and one N-free diet (e.g., Trindade Neto et al., 2012; Woyengo et al., 2016b). The N-free diet served a dual purpose: calculation CSID of AA and CAID and CATTD of energy of test ingredients. Furthermore, the wheat grain provided more fermentable material than canola oil, sugar and canola oil thereby supporting comparisons of fermentability between low protein (wheat) diet with diets with increasing CM and thus protein. Therefore, these formulations provided information on effects of CM and its inclusion level on digestibility of energy and AA and hindgut fermentation in growing pigs.

Using a specific variety of CM requires evaluation of their nutrient digestibility and fermentability in growing pigs. The study showed that *B. juncea* CM had superior quality including greater fermentability, greater CATTD of energy and thus greater DE value than *B. napus* CM although digestibility of amino acids did not differ. Interestingly, the findings also revealed that *B. juncea* CM may limit protein fermentation in the pig intestine due to its fermentation characteristics. Increasing inclusion of *B. napus* CM increased the molar ratios of branched-chain fatty acids in pig feces, which indicates greater protein fermentation (Macfarlane et al., 1992). In contrast, increasing inclusion of *B. juncea* CM reduced the molar ratios of branched-chain fatty acids in feces of growing pigs. It suggests a shift from protein fermentation to carbohydrate fermentation in the hindgut of pigs as a result of greater concentration of fermentable carbohydrates in *B. juncea* CM. Inclusion of fermentable carbohydrates in weaned pig diets is a promising approach to modulate the composition of microbiota along the gastrointestinal tract and thereby improve pig health (Pieper et al., 2012; Heo et al., 2013; Rist et al., 2013).

Furthermore, feeding *B. juncea* or *B. napus* CM to pigs potentially stimulate bacterial growth differently along the gastrointestinal tract due to their substrate preference to specific fermentable NSP (Metzler-Zebeli et al., 2010). Understanding the fermentability of each type of canola co-products may benefit pig health and increase feeding value of the canola co-products. Fermentation of dietary pectin starts at the ileum and promotes the growth of *Bacteroides-Prevotella-Porphyromonas* at this section of growing pigs (Metzler et al., 2009). The molar ratio of acetate was not different between canola species in ileal digesta, whereas it was greater in feces of pigs fed *B. juncea* CM. Greater concentration and proportion of butyric acid in ileal digesta could be associated with greater content of xylose in *B. napus* CM than *B. juncea* CM (9.1 vs 7.5%, as DM basis), which contributed to greater butyric acid production in the ileum (Slominski et al., 2012; Ivarsson et al., 2014).

5.2. Extruded *B. juncea* canola expeller

Inclusion of *B. juncea* canola expeller in weaned pig diets established in chapter 3 that ADFI and ADG curved at 240 g extruded *B. juncea* canola expeller/kg likely because increased dietary glucosinolate intake that may prevent further increases in ADFI. The study also indicated that diet formulation based on NE and SID AA systems reduced risks associated with feeding high-fiber, high-protein co-products (Zijlstra and Beltranena, 2013). Experimental diets were formulated using the NE value of canola oil published in NRC (1998) that was lower than the updated value (NRC, 2012). As a result, NE values of diets decreased, and pigs fed increased inclusion of extruded *B. juncea* canola expeller consumed more feed to meet their requirements. It showed that weaned pigs might be fed up to 180 g/kg *B. juncea* canola expeller without reducing growth performance. At this inclusion level, dietary glucosinolate content was 1.96

μmol/g, which is still below the maximal tolerance of pigs that was identified at 2.5 μmol/g (Schone et al., 1997).

5.3. Fermented wheat with *L. reuteri*

In addition to including co-products into swine diets, feeding fermented feed is another feeding strategy to improve health and performance of young pigs. In chapter 4, feeding fermented wheat with *L. reuteri* provided benefits of prebiotics, exopolysaccharides, to newly weaned pigs on development of intestinal villi and production of SCFA. Weaned pigs fed fermented wheat grain diets had reduced abundance of enterotoxigenic *E. coli* in colonic digesta and feces (Chapter 6, Appendix). In addition, copy numbers of genes of *E. coli* and heat-stable enterotoxin were not detected in the ileum, cecum and colon of pigs fed fermented-reuteran-containing diets (Yang et al., 2015). Exopolysaccharides in fermented wheat, reuteran and levan, exerted prebiotic effects by favoring the growth of commensal flora *B. thetaiotaomicron* with increased concentrations of SCFA that could contribute to the reduction of *E. coli* in weaned pigs. The diversity of intestinal microbiota increased over time but *Bacteroidetes* and *Firmicutes* remained dominant, as evidenced by Leser et al. (2002).

5.4. Challenges and limitations of studies

One of the challenges in this thesis is that the model to study nutrient digestibility of two CM was the growing pigs (> 30 kg body weight) instead of the weaned pig (8-20 kg body weight). Therefore, it might be not accurate to apply the digestibility data measured with growing pigs to weaned pigs, because nutrient digestibility depends on the age and growth stage of pigs (Bell and Keith, 1989). Furthermore, the lack of using the ileal T-cannulation model in weaned pigs

prevented us from collecting sufficient ileal digesta to analyze ileal digestibility in the third experiment. Collection of ileal digesta by slaughter technique was limited in obtaining insufficient amount of ileal digesta for chemical analyses (Donkoh et al., 1994).

There were some challenges to feeding fermented wheat grain diets. First, fermented wheat diets were prepared without feed flavour and taste enhancer to attract piglets to eat. The sour taste might explain the lower feed intake of young piglets fed fermented wheat diets compared with pigs fed control and acidified diets. Thus, reduced feed intake may lessen the benefits of fermented wheat grain diets on intestinal development. Addition of sweeteners such as sugar, molasses, and artificial sweeteners, might increase palatability of fermented wheat diets (Dong and Pluske, 2007; Clouard et al., 2012). Second, fermented wheat grain diets in the study were wet and a little bit sticky; thus, they were more difficult to chew by the piglets. It might be more attractive to piglets if these diets were more liquid (Russell et al., 1996). Finally, the study was conducted on healthy weaned pigs without diarrhea during the course of experiment; thus, we were unable to test *in vivo* effects of feeding exopolysaccharides on preventing diarrheal disease and associated reduced weight gain in newly-weaned pigs.

Growth performance data of weaned pigs presented in the Appendix were different from those in Chapter 4 because of statistical analyses. The SAS code used in Chapter 4 to analyse growth performance included diet × week interactions and contrasts to compare combinations of different treatments within each week and for the overall trial. This approach also allowed comparison and selection of the best variance-covariance structure for analysis of repeated measures. Consequently, the SAS code used in Chapter 4 provided more accurate data of weekly and overall growth performance.

5.5. Future research

Glucosinolates in *B. juncea* CM have been known to reduce feed intake in young pigs and cause adverse effects on their growth performance. However, the metabolism of glucosinolates in the gastrointestinal tract of pigs needs to be clarified to take advantages of microbial fermentation in the gut, especially in pigs fed fermented feed. Furthermore, fermentation of canola co-products needs to be studied to take advantages of their high fiber content. The inconsistent nutritional values of canola co-products due to their processing methods could be considered (Woyengo et al., 2016a, b).

It is essential to study nutrient digestibility in weaned pigs using T-cannula to better and more accurate understand of nutrient utilization in feedstuffs. At present, limited data exist on T-cannula application in weaned pigs because the small intestinal diameter makes it more difficult to insert a thick-walled plastic cannula without blocking digesta flow (Walker et al., 1986). Digesta collection from the opened cannula allows the collection of more digesta than by using the slaughter technique and therefore a better assessment of ileal digestibility of nutrients at multiple time points rather than once at the end of the study using the slaughter technique. The T-cannulation technique also provides opportunities to test more feed ingredients in weaned pigs.

Fermentation patterns of microbial communities in the hindgut of weaned pigs need to be measured to modify feedstuff utilization in diets and to improve gut health then growth performance. Delivery of fermented feed and palatability also need to be improved and made applicable to individual farms. Research in combination of canola meal and fermented wheat with *L. reuteri* and exopolysaccharides in diets of healthy or unhealthy weaned pigs might bring more profits to swine producers when maximizing the use of available co-products without compromising pig health and growth.

5.6. Practical implications of the studies

In swine production, feed contributes approximately 70% of production cost (Mullan et al., 2011). The comparable values of nutrient digestibility of *B. juncea* to *B. napus* CM revealed benefits of using *B. juncea* in swine diets in terms of energy value of the meal. This finding increases the chance of using more types of canola meal for pigs, thus providing more flexibility in feed formulation. Besides, some characteristics of the *B. juncea* crop benefit growers in rotations with cereals and expansion of their oilseed production, include greater tolerance to heat and drought, greater resistance to diseases, earlier maturation and less seed shattering at maturity than conventional *B. napus* (Johnston et al., 2002; Gan et al., 2007).

Inclusion of up to 180 g extruded *B. juncea* expeller/kg diet did not affect growth performance of weaned pigs but increased profitability (Woyengo et al., 2014). Assuming that the prices of wheat, soybean meal, extruded *B. juncea* expeller, canola oil, and L-lysine-HCl were $207, $402, $250, $1300, and $2550 per metric tonne, respectively, feed price reduced by $42/ton and because growth performance was maintained, feed cost decreased ¢7 per kg of body weight gain. Additionally, feeding fermented feed with *L. reuteri* and exopolysaccharides demonstrated beneficial of prebiotic effects on small intestinal development and hindgut fermentation in weaned pigs. The research included in this thesis therefore offers potential feeding strategies that promote health benefits, prevent enteric diseases, and manage growth of weaned pigs, thus bring economic profitability to swine producers. Newly weaned pigs can be fed diets formulated on-farm from local feedstuffs, fermented wheat grains with canola co-products. It helps reduce feed cost, promote gut health and improve growth performance in weaned pigs.

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APPENDIX – Feed fermentation with reuteran- and levan-producing *Lactobacillus reuteri* reduces colonization of weanling pigs with enterotoxigenic *Escherichia coli*

Abstract

This study determined the effect of feed fermentation with *Lactobacillus reuteri* on growth performance and abundance of enterotoxigenic *Escherichia coli* (ETEC) in weanling piglets. *L. reuteri* strains produce reuteran or levan, exopolysaccharides that inhibit ETEC adhesion to the mucosa, and feed fermentation was conducted under conditions supporting exopolysaccharide formation and under conditions not supporting exopolysaccharide formation. Diets were chosen to assess the impact of organic acids, and the impact of viable *L. reuteri* bacteria. Fecal samples were taken throughout 3 weeks of feeding; at the end of the 21-day feeding period, animals were euthanized to sample the gut digesta. The feed intake was reduced in pigs fed diets containing exopolysaccharides; however, feed efficiency did not differ among the diets. Quantification of *L. reuteri* by quantitative PCR (qPCR) detected the two strains used for feed fermentation throughout the intestinal tract. Quantification of *E. coli* and ETEC virulence factors by qPCR demonstrated that fermented diets containing reuteran significantly ($P < 0.05$) reduced the copy numbers of genes for *E. coli* and the heat-stable enterotoxin in feces compared to those achieved with the control diet. Any fermented feed significantly ($P < 0.05$) reduced the abundance of *E. coli* and the heat-stable enterotoxin in colonic digesta at 21 day; reuteran-containing diets reduced the copy numbers of the genes for *E. coli* and the heat-stable enterotoxin below the detection limit in samples from the ileum, the caecum, and the colon. In conclusion, feed fermentation with *L. reuteri* reduced the level of colonization of weaning piglets with ETEC, and

feed fermentation supplied concentrations of reuteran that may specifically contribute to the effect on ETEC.

Introduction

The combined stress of weaning and movement to a different environment increases the potential for poor growth performance, nutrient malabsorption, and diseases in piglets (1–3). Diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC) is a major disease of weaning piglets (4). ETEC establishes infection by specific fimbria mediating intestinal attachment and colonization (4–7). Following colonization, ETEC produces enterotoxins that induce watery diarrhea (8, 9). Control of ETEC infections of weanling piglets is currently achieved by antibiotics (10). The use of antibiotics in animal production, however, selects for antibiotic-resistant intestinal microbiota and favors the transfer of antibiotic resistance genes from livestock microbiota to human pathogens (7). Consequently, antimicrobial growth promoters have been banned in several jurisdictions (11), challenging the livestock industry to replace feed antibiotics without compromising animal performance or animal health.

Probiotic bacteria, prebiotics, organic acids, or anti-adhesive glycans were proposed to replace feed antibiotics in pig production for improved control of ETEC (4, 6). Organic acids were shown to reduce postweaning diarrhea in pigs (12). Probiotics also decreased the incidence and severity of ETEC-caused diarrhea (13, 14). Neoglycans of porcine albumin conjugated with galacto-oligosaccharides reduced ETEC attachment *in vitro* (15). ETEC adhesion *in vitro* was also reduced by reuteran and levan, exopolysaccharides produced by *Lactobacillus reuteri* (16). The protective effect of reuteran was confirmed *in vivo* in the small intestinal segment loop perfusion model (17). Evidence for the effectiveness of probiotic cultures or anti-adhesive

glycans for the prevention of ETEC infection *in vivo*, however, remains limited. Moreover, a combination of different additives is likely required to obtain effective and economically viable alternatives for feed antibiotics (18, 19). Feed fermentation can deliver a combination of viable and probiotic lactobacilli, organic acids, and exopolysaccharides that prevent pathogen adhesion (20).

L. reuteri is a member of the commensal microbiota in swine (21, 22); it is also used industrially as a starter culture in cereal fermentations (23). *L. reuteri* converts maltose and glucose to lactic and acetic acids; sucrose is converted to the alternative end products organic acids, mannitol, oligosaccharides, or exopolysaccharides (24). The growth of specific strains of *L. reuteri* in cereal substrates also supports the formation of reutericyclin, an antibiotic with specific activity against Gram-positive pathogens (25, 26). Moreover, the exopolysaccharides reuteran and levan, which prevent adhesion of ETEC K88 fimbriae to the porcine intestinal mucosa (16, 17), are produced during the growth of *L. reuteri* in cereals (27, 28). However, the specific contribution of exopolysaccharide formation on the inhibition of intestinal pathogens by *L. reuteri* remains unknown. This study therefore aimed to determine the effect of feed fermentation with *L. reuteri* on the growth performance as well as the abundance of intestinal ETEC organisms in weanling piglets. Strains of *L. reuteri* were chosen to include the reuteran-producing strain *L. reuteri* TMW1.656 and the levan producing strain *L. reuteri* LTH5794 (16). Fermented and chemically acidified feed served as control to differentiate between the effects of organic acids and those of viable *L. reuteri* organisms. To identify the specific effects of exopolysaccharide formation, feed was fermented with addition of 10% sucrose to support reuteran or levan formation by *L. reuteri* or without sucrose addition to obtain the same cell counts and the same concentration of organic acids but no bacterial exopolysaccharides.

Past studies to determine the effect of feed additives used ETEC-challenged pigs (13, 29, 31). This study employed piglets that were housed and fed under conditions that are close to those used in industrial practice but that were not challenged with ETEC. This approach allowed investigation of the effect of fermentation on the diverse ETEC strains that are present in unchallenged piglets and to assess the effect of feed fermentation on animal performance, in addition to its effect on animal health.

Materials and Methods

Microorganisms and growth condition

L. reuteri TMW1.656 and *L. reuteri* LTH5794, which produce reuteran and levan, respectively, from sucrose (16), were routinely grown on modified MRS agar (32) and incubated anaerobically at 37 °C for 48 h. To obtain working cultures for feed fermentation, colonies were subcultured twice in modified MRS broth. *E. coli* strain ECL13795 (O149; virotype STb:LT:EAST1:F4) (17) was used as a positive control to determine the specificity of primers targeting *E. coli* and *E. coli* virulence factors.

Optimization of reuteran and levan production in feed fermentations and feed fermentations

To optimize conditions for reuteran and levan formation, white wheat and corn flour (provided by the University of Alberta Swine Research and Technology Centre [SRTC]) were mixed with an equal amount of tap water. Sucrose was added at 10 or 20% (weight sucrose/weight flour). Cells from overnight cultures of *L. reuteri* were washed with sterile tap water and added at cell counts of approximately 10^7 CFU g^{-1}. After 24 h of fermentation at 37°C, samples were taken and the pH, cell counts, concentrations of organic acids and ethanol, and concentrations of

reuteran and levan were determined as previously described (33). In brief, organic acids and ethanol were extracted from fermented feed and quantified by high-pressure liquid chromatography after separation on an Aminex HPX-87 column and detection on a refractive index detector (33). Reuteran and levan were extracted by aqueous extraction from freeze-dried samples, dialyzed against distilled water, and quantified by size exclusion chromatography (33).

For feed fermentations, a seed sourdough was prepared with ground wheat and 10% sucrose as described above and transported to the SRTC. Ground wheat was prepared with wheat of the variety Harvest HRS (2012 harvest year), which was ground through the weaned pig screen (size 3/32). The seed sourdough was used to inoculate the first feed fermentation with a 10% inoculum. After 24 h of fermentation, 90% of the batch was used to feed the piglets; the remaining 10% was used to inoculate the subsequent batch of fermented feeds. After four fermentation cycles with 10% inoculum and 24h of fermentation each, seed sourdoughs were prepared in the laboratory from the culture stock and used to inoculate the feed fermentations. Wheat fermented with the same strains and addition of 5% (wt/wt) glucose and 5% (wt/wt) fructose in place of sucrose served as exopolysaccharide-negative controls. A chemically acidified control was prepared with 5% (wt/wt) fructose, 5% (wt/wt) glucose, 4 parts of lactic acid (80%), and 1 part of glacial acidic acid (100%) to reach a pH of 3.8.

To verify that the strains used to inoculate fermented feed dominated the fermentation, the pH of each batch was measured after 24 h of fermentation. The cell counts in each batch of fermented feed were determined by serial dilutions and surface plating on MRS agar plates. All colonies on the modified MRS plates exhibited a uniform colony morphology that matched the colony morphology of the inoculum. In sourdoughs started with defined strains of lactobacilli, a

matching and uniform colony morphology is a reliable measure for the absence of contaminants (34).

Animals and diets

The animal study was approved by the Animal Care and Use Committee of the University of Alberta according to the guidelines of the Canadian Council on Animal Care and was approved to be conducted at the SRTC. A total of 36 crossbred castrated male piglets were selected at weaning at 21 days of age. Each piglet was housed in an individual pen (0.5 by 1.22 m) in a temperature-controlled room (28 ± 2.5 ºC) during the 3-week experiment.

The piglets were randomly divided into six blocks containing six piglets each. One piglet per block was assigned to one of six experimental diets for a total of six observations per diet. Wheat or fermented wheat was included in the diets as follows: diet 1, unfermented wheat; diet 2, unfermented wheat acidified to pH 3.8 with lactic acid and acetic acid and supplemented with 5% glucose and 5% fructose; diet 3, wheat fermented with *L. reuteri* TMW1.656 and supplemented with 10% sucrose to support reuteran production; diet 4, wheat fermented with *L. reuteri* TMW1.656 and supplemented with 5% glucose and 5% fructose, which do not support reuteran formation; diet 5, wheat fermented with *L. reuteri* LTH5794 and supplemented with 10% sucrose to support levan production; diet 6, wheat fermented with *L. reuteri* LTH5794 and supplemented with 5% glucose and 5% fructose, which do not support levan formation. The diet was formulated to meet or to exceed National Research Council (NRC, 2012) nutrient recommendations for 5- to 10-kg pigs (Table 1). Two phases of the diets were fed sequentially within each treatment. From day 0 to day 6, 20% fermented wheat was added to the basal diet (phase 1 diet); from day 7 to 21, the proportion of fermented wheat in the diet was increased to 50% (phase 2 diet). Titanium dioxide (TiO_2) was added as an indigestible marker to each of the

test diets to calculate total tract digestibility coefficients of the nutrients. Piglets were offered free access to feed from a pen feeder and water from a nipple drinker. They were fed in mash form twice daily, at 8 a.m. and 4 p.m. At the end of the trial, piglets were fed their final meal 3 to 4 h before being euthanized to ensure that the digesta had reached each section of the small intestine. Body weight was recorded on days 0, 7, 14 and 21. Feed intake was measured each day. All of the data were used to determine average daily weight gain (ADG), average daily feed intake (ADFI) and feed efficiency (ratio of weight gain to feed intake [G/F]).

Sample collection and preparation

Fresh feces were collected in a plastic bag by hand grabbing of the feces from the floor of each pen on days 0, 7, 14 and 21 and stored at −20°C. After the piglets were euthanized, gut digesta were collected from the stomach, jejunum, ileum, cecum and mid-colon and placed in sterile plastic containers. Samples were stored at −20°C.

Frozen fecal and gut digesta samples were thawed and mixed aseptically with a spatula. For bacterial analysis, two 1.0- to 1.5-g subsamples were taken and stored at −80°C. Tissue samples from the jejunum and ileum (about 5 cm) were aseptically excised. The segments were opened longitudinally, and the mucosa was removed by scraping with a flame-sterilized metal spatula (35). The mucosal scrapings were stored in individual tubes at −80°C for bacterial analysis.

Genomic DNA extraction for quantitative PCR (qPCR)

Total bacterial DNA was extracted from fecal and gut digesta samples using QIAamp®DNA stool minikit (Qiagen, Inc., Valencia, CA, USA) (36) following the manufacturer's instructions. Briefly, about 120 mg (wet weight) of fecal sample was homogenized in buffer ASL (Qiagen) and heated at 95°C for 5 min to lyse the bacterial cells. After centrifugation 20,000 × *g* for 1 min

at room temperature (approximately 20°C), the supernatant was incubated with an InhibitEx tablet to absorb DNA-damaging compounds and PCR inhibitors (QIAamp DNA stool mini kit handbook). Proteins in the lysates were removed by treatment of the samples with proteinase K and buffer AL (Qiagen) at 70°C for 10 min. Ethanol (96-100%) was added to the lysate to precipitate the DNA, and the mixture was applied to the QIAamp spin columns provided in the kit. The columns were washed with buffers AW1 and AW2 (Qiagen), and the DNA was eluted in buffer AE (Qiagen).

The DNA concentration was measured in a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific Inc., Wilmington, NC, USA), and the DNA purity was assessed by determining the ratio of absorbance at 260 to the absorbance at 280 nm. All DNA samples had a 260 nm/280 nm absorbance ratio of > 1.8.

PCR primers

The primers used in this study and their target organisms are listed in Table 2. Primers GTFA F and GTFA R are specific for the detection of *gtfA,* the gene coding for reuteransucrase in *L. reuteri* TMW 1.656. To obtain primers that specifically detect the gene coding for reutericyclin biosynthesis in *L. reuteri* TMW1.656, primer RC F and RC R were designed in Primer 3 software (37) to target the *rtcN* gene. *rtcN* codes for a nonribosomal peptide synthase; the gene is essential for reutericyclin biosynthesis but is essentially absent in all other lactobacilli (GenBank accession number KJ659887.1) (26). To obtain primers for the specific detection of the gene coding for levansucrase in *L. reuteri* LTH5794, *ftfA,* primers FTF F and FTF R were designed in Primer 3 software (37) to target the gene coding for a levansucrase in *L. reuteri* SD2112 (GenBank accession number NC_015697). The PCR amplicons that were obtained with chromosomal DNA of *L. reuteri* LTH5794 and *L. reuteri* TMW1.656 as the template were

purified from agarose gels and sequenced by Sanger sequencing (Macrogen, Rockville, MD). The sequence data were aligned with ClustalW program (38) to identify sequences that are unique to *ftfA* of *L. reuteri* LTH5794. These unique sequences were used to design primers LEV F and LEV R. The Basic Local Alignment Search Tool (BLAST) was initially used to determine the specificity of the primer sequences (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The specificity of the primers was subsequently verified in qPCRs with DNA from *L. reuteri* TMW1.656 or LTH5794 as the template, followed by determination of the sizes and melting temperatures of the amplicons (Fig. 1).

Primers were synthesized by Integrated DNA Technologies Inc., diluted to a final concentration of 10 μg of primer per μl with autoclaved Milli-Q water upon receipt, and stored at −20°C.

Quantification of bacteria and bacterial metabolites or toxins by qPCR

qPCR was performed on a 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The total reaction volume of 20 μl contained 10 μl of QuantiFast SYBR green master mix (Applied Biosystems), 2 μl (10 μM) of primers, and 1 μl of template DNA from fecal or gut digesta samples. Each reaction was run in duplicate in a MicroAmp Fast Optical 96-well reaction plate sealed with MicroAmp optical adhesive film (Applied Biosystems). The concentration of template DNA was about 100 mg liter^{-1}. PCR amplicons were purified by using QIAquick PCR purification kit (250), according to the manufacturer's instructions.

Standard curves were generated using serial 10-fold dilutions of the purified PCR amplicons, which were amplified from PCRs with the same primers (Table 2) and genomic DNA from *L. reuteri* strains or pig feces. The initial concentration of the purified PCR amplicons was determined by use of a NanoDrop spectrophotometer. Amplification conditions generally

involved 1 cycle at 95°C for 5 min for initial denaturation, 40 cycles of denaturation at 95°C for 30 s, annealing with optimal annealing temperatures (Table 2) for 30 s, and extension at 72°C for 30 s. In the melting curve stage, the reaction conditions included 1 cycle of 95°C for 15 s, 1 cycle at 60°C for 1 min, and a stepwise increase of the temperature from 55 to 95°C (at 10 s per 0.5°C). To insure correct amplification results, the melting curves were checked to verify that the PCR amplicons yielded a single melting peak.

Statistical analysis

Data were analyzed using Statistical Analysis System (SAS; version 9.3) software (SAS Institute, 2012). Data for fecal bacteria and growth performance were analyzed according to a randomized complete block design with repeated measurement using the mixed procedure (Proc MIXED). The model included diet, period and the interaction of diet and period as fixed effects. The blocks were considered random effects, and the individual animals were considered experimental units. Data for the microbiota of the gut digesta were subjected to analysis of variance as a randomized complete block design using the general linear model procedure (Proc GLM). Treatment comparisons were determined by contrast. The experimental unit was the piglet, and differences with a *P* value of <0.05 with a Bonferroni adjustment for multiple comparisons (SAS version 9.3) were considered to be statistically significant. The Kolmogorov-Smirnoff test (39) was used to test for normality of all variables. The results of growth performance analyses are presented as means, while data from analyses of the bacteria are presented as least-squares means with standard errors.

Results

Production of reuteran and levan in feed fermentations

Reuteran and levan formation in feed fermentations was evaluated with wheat and corn. The *in situ* production of reuteran or levan by *L. reuteri* TMW1.656 in corn fermentation was low compared to that in wheat fermentation with the same sucrose addition (data not shown). Therefore, ground wheat was used for feed fermentations.

The levels of metabolite formation after fermentation by *L. reuteri* in wheat are shown in Table 3. The highest reuteran yield from *L. reuteri* TMW1.656 was obtained in fermentations with addition of 10% sucrose (Table 6.3). Addition of 10% sucrose provided fermented feed with lower acetate concentrations than addition of 20% sucrose. Because acetic acid may reduce the palatability of fermented feed, wheat flour with addition of 10% sucrose was used to obtain fermented feed.

The six diets used in the present study were chosen to assess the impacts of organic acids (control versus chemically acidified feed), of viable *L. reuteri* (unfermented controls versus four fermented diets), and of reuteran and levan (fermented diets supplemented or not supplemented with sucrose to support reuteran and levan formation).

Growth performance of pigs and animal health

Data indicating the growth performance in the 21-day trial are presented in Table 6.4. Pigs fed diets containing reuteran or levan displayed a reduced average daily feed intake and a reduced average daily weight gain compared with those for pigs fed unfermented diets. The average daily feed intake was lower in pigs fed fermented diets than pigs fed unfermented diets. However, feed efficiency did not differ among the diets. All pigs remained healthy throughout the experimental period and did not develop diarrhea.

Genus-, species-, and strain-specific detection of lactobacilli in fecal samples

qPCR with strain-specific primers were employed to determine whether the strains employed for feed fermentation remained present throughout the gastrointestinal transit. Organisms of the *Lactobacillus* group and *L. reuteri* were quantified to determine whether the dietary *L. reuteri* influenced the abundance of autochthonous lactobacilli and *L. reuteri*. The genes coding for reuteransucrase and levansucrase in *L. reuteri* TMW1.656 and LTH5794, *gtfA* and *ftfA*, respectively, were used as strain-specific markers. The copy numbers of the genes representing the *Lactobacillus* group remained stable or increased slightly during the study period (Table 6.5). The copy numbers of the genes representing *L. reuteri* increased after 7 days of feeding in all groups; with the exception of piglets fed chemically acidified diets, the fecal copy numbers of the genes representing *L. reuteri* decreased again at day 21. In animals fed chemically acidified diets, *L. reuteri* numbers were maintained at the same high level from day 7 to day 21. At days 14 and 21, *L. reuteri* numbers in piglets that were fed diets fermented with *L. reuteri* TMW1.656 were lower than those in the group fed chemically acidified diets.

Before the treatments (day 0), *gtfA* or *rtcN* was not detected in any sample (Table 6.5), while *ftfA* was detected only in pigs assigned to the control group. The gene for reuteransucrase and *rctN* was detected at days 7, 14, and 21 in pigs receiving feed fermented with *L. reuteri* TMW1.656 and at lower copy numbers in pigs fed chemically acidified diets. The copy numbers of the *ftfA* gene of *L. reuteri* LTH5794 were 2 log units higher than the copy numbers of the gene in the feces of pigs fed other diets; however, the copy numbers of *ftfA* were above the detection limit in piglets fed the unfermented control diet at all times and in piglets of the other groups at day 14.

Genus-, species-, and strain-specific detection of lactobacilli in gut samples

Lactobacilli were quantified in gut samples at the day 21. The copy numbers of the gene representing the *Lactobacillus* group were high in all intestinal compartments except the ileum, where the numbers were 1 to 2 log(gene copies g^{-1}) lower (data not shown). The copy numbers of the gene representing *L. reuteri* in the stomach, the ileum, and the cecum were higher for piglets fed fermented diets than piglets fed chemically acidified diets (data not shown); however, the copy numbers of the gene representing *L. reuteri* in the colon of animals fed *L. reuteri* TMW1.656 were reduced (data not shown).

The copy numbers of the *gtfA* gene of *L. reuteri* TMW1.656 were high in all samples from piglets that were fed *L. reuteri* TMW1.656, and the copy numbers of the *gtfA* gene were low or the gene was absent in samples from other piglets (Fig. 6.1). Matching results were obtained with primers targeting *rtcN*, the second strain-specific marker for *L. reuteri* TMW1.656 (Fig. 6.2). *ftfA* of *L. reuteri* LTH5794 was detected only in pigs that were fed diets fermented with this strain (Fig. 6.3).

Detection of *E. coli* and ETEC virulence factors in fecal samples

E. coli and ETEC were quantified by qPCR to determine the effect of feed fermentation and reuteran or levan production by *L. reuteri* on the numbers of intestinal *E. coli* bacteria (Table 6.6). *E. coli* and ETEC were detected in feces of all pigs at all times. The copy numbers of genes representing *E. coli* decreased over time in animals that were fed fermented diets but not in animals fed the control diet or the chemically acidified diet. At day 21, the gene copy numbers of genes representing *E. coli* were the lowest in animals receiving fermented diets containing reuteran. The levels of the genes for virulence factors of ETEC, heat-stable enterotoxin b (STb), heat-labile enterotoxin (LT), and K88 fimbriae, peaked at day 7 or day 14 in all animals and

decreased at day 21. The copy numbers of the gene for STb were lower in animals receiving fermented diets containing reuteran than in the control animals or animals that were fed the chemically acidified diet. Moreover, samples from animals receiving reuteran were the only samples where the copy numbers of the gene for LT were below the detection limit. The copy numbers of the gene for K88 fimbriae were below the detection limit in all animals except animals in the control group, or the group receiving feed fermented with *L. reuteri* LTH5794 but without sucrose.

Detection of genes coding for *E. coli* and ETEC virulence factors in gut samples

Genes coding for *E. coli* and STb were detected in the gut digesta of pigs fed unfermented diets; in contrast, the copy numbers of the genes for *E. coli* and STb were below the detection limit for all samples from pigs fed a reuteran-containing diet fermented with *L. reuteri* TMW1.656 (Fig. 6.3). In animals fed *L. reuteri* LTH5794, the gene copy numbers for *E. coli* and ETEC were below the detection limit in ileal samples; the gene copy numbers in the colon were lower than those in samples from animals fed unfermented diets. Other virulence factors of ETEC, including LT, heat-stable enterotoxin a (STa), and K88 fimbriae, were not detected in any of the samples from gut digesta (ileum, cecum, and colon). Neither *E. coli* nor its virulence factors were detected in samples of mucosal scrapings from the jejunum.

Discussion

ETEC causes diarrhea in newborn and weaned pigs, resulting in serious morbidity and mortality and major financial losses in the swine industry (6, 40). The present study demonstrated that feed fermentation with two exopolysaccharide-producing strains of *L. reuteri* reduced the abundance of ETEC in weanling piglets. Beneficial effects of feed fermentation related predominantly to the

ingestion of viable cells of *L. reuteri*. In addition, the presence of reuteran produced during feed fermentation further reduced the numbers of ETEC bacteria.

Fermented feeds may benefit gut health and improve the growth performance of pigs (20, 41). In previous studies, the average daily weight gain and the average daily feed intake of pigs fed fermented liquid diets were reduced compared to those of pigs receiving unfermented feed; however, feed efficiency did not differ (41, 42). The present study confirmed the reduced feed intake and unchanged feed efficiency in pigs receiving fermented feed. Remarkably, this effect was less pronounced in the groups that received fermented feed without reuteran or levan. The formation of reuteran and levan from sucrose reduces the sweet taste of the feed because sucrose is converted to oligosaccharides and polysaccharides that do not taste sweet (24, 43). In fermented or unfermented control feeds supplemented with glucose and fructose, the levels of acidity are comparable but the concentrations of monosaccharides and mannitol are higher. Pigs thus might prefer a balance of sweet and sour tastes. This finding suggests the possibility that feed intake may be increased through the formulation of feeds with the aim of improving their taste.

Organic acids promoted the growth of weaning pigs due to their antimicrobial properties and because they lower the gastric pH (12, 44); however, the present study did not reveal positive effects of organic acids on growth performance or inhibition of *E. coli*. The abundance of lactobacilli and *L. reuteri* increased in animals fed chemically acidified diets. Chemically acidified feed was supplemented with glucose and fructose so that the sugar levels matched those in the fermented diets and thus provided more fermentable substrates to autochthonous lactobacilli in the stomach (45).

qPCR is widely used to detect bacteria in fecal and gut content samples (46). Because the viable *L. reuteri* that are present in the feed or the upper intestinal tract remain viable throughout gastrointestinal transit (21, 28, 47), quantification of DNA by qPCR indicates the presence of viable cells. The primers were designed to target strain-specific sequences in *L. reuteri* TMW1.656 and *L. reuteri* LTH5794, and the melting temperature of amplicons was routinely checked to verify specific amplification. Cross-contamination was essentially absent in intestinal samples. However, low levels of *gtfA* and *ftfA* were detected in fecal samples from pigs that were not fed the corresponding strains. This may reflect the cross contamination of fecal samples after defecation. Despite this limitation, the qPCR methodology differentiated between feed-fermenting *L. reuteri* strains and autochthonous strains of lactobacilli (21, 23). Both strains persisted in the gut of the piglets, were excreted with feces in high abundance, and accounted for 1% to > 10% of the total *L. reuteri* bacteria. *L. reuteri* TMW1.656 and LTH5794 are rodent lineage strains that differ physiologically and phylogenetically from porcine strains of *L. reuteri* (21, 23). *L. reuteri* LTH5794 had no apparent influence on autochthonous lactobacilli, in keeping with the findings of previous studies feeding probiotic lactobacilli (47, 48). Feed fermented with *L. reuteri* TMW1.656, however, decreased the abundance of autochthonous *L. reuteri* bacteria. *L. reuteri* TMW1.656 produces reutericyclin (25), a low-molecular-weight antimicrobial compound inhibiting Gram-positive bacteria, including *L. reuteri* (49), that may account for the reduced numbers of *L. reuteri* bacteria.

Oral administration of lactic acid bacteria to swine reduced the amounts of fecal *Enterobacteriaceae* and coliform bacteria (47); oral administration of *L. amylovorus* also reduced the level of colonization of weaning piglets with ETEC in a challenge study (36). The present study demonstrated that feed fermentations reduce the abundance of intestinal *E. coli*,

including ETEC, compared to that achieved with both the unfermented control and the acidified unfermented control. Although all piglets remained healthy throughout the study, a reduced number of ETEC bacteria indicates a reduced risk of ETEC-induced diarrhea. This health benefit justifies the designation of the two strains as probiotic strains (50).

The effect of feed fermentation with *L. reuteri* on the abundance of fecal *E.coli* or fecal levels of ETEC virulence factors was dependent on the strain and on the presence of reuteran or levan. The reuteran-containing fermented diet was the only diet that significantly reduced the fecal levels of STb compared to those in both unfermented control diets; this diet was also the only diet reducing the levels of fecal LT to levels below the detection limit at week 3 and reducing the levels of *E. coli* and STb to levels below the detection limit in all intestinal tissue samples (Table 6 and Fig. 3). Exopolysaccharides produced by lactic acid bacteria reduced the adherence of pathogenic bacteria, such as *E. coli*, to the intestinal mucosa (51). Specifically, the reuteran produced by *L. reuteri* TMW1.656 reduces the adhesion of ETEC K88. In the present study, the reuteran-positive groups had lower copy numbers of genes for *E. coli* and the ETEC virulence factors than groups fed diets containing *L. reuteri* but not reuteran or levan, which may reflect the anti-adhesive properties of reuteran *in vitro* (16) and in an *in vivo* model (17). Together with the findings presented in those prior reports, this study suggests that reuteran reduced the level of ETEC adhesion to the intestinal mucosa *in vivo.* However, the levels of *E. coli* and ETEC bacteria in animals receiving *L. reuteri* TMW1.565-fermented diets with or without reuteran were not significantly different. This may relate to the low levels of reuteran that may be formed from the sucrose that is present in wheat flour (43).

Conclusion

In conclusion, feed fermentation with *L. reuteri* reduced the level of colonization of weaning piglets with ETEC bacteria and additionally supplied reuteran in concentrations that may specifically contribute to the prevention of ETEC adhesion to the intestinal mucosa. The study thus constitutes a step towards understanding of the metabolic activities that confer probiotic properties to lactic acid bacteria.

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Table 6.1. Composition of experimental diets on an as-fed basis

	Composition (%)	
Ingredient	Phase 1 diet (days 0 to 6)	Phase 2 diet (days 7 to 21)
Ground and fermented or unfermented wheat	20.00	50.00
Corn	31.54	1.76
Lactose	15.00	10.00
Soy protein concentrate	3.00	2.50
Herring meal	6.00	2.50
Brassica napus canola meal		5.00
Wheat DDGS[a]		5.00
Soybean meal	15.00	15.00
Canola oil	4.00	3.40
Other vitamin and mineral ingredients	5.46	4.84
Total	100	100

[a] DDGS, distillers dried grains with solubles.

Table 6.2. Primers used to profile the microorganisms in fecal and gut digesta samples

Target	Primer	Sequence (5'-3')	Product size (bp)	T_m [b] (°C)	Reference or source
Lactobacillus group[a]	Lacto F	TGGAAACAGRTGCTAATACCG	231-233	64	53
	Lacto R	GTCCATTGTGGAAGATTCCC			
L. reuteri	Lreu F	CAGACAATCTTTGATTGTTTAG	303	64	54
	Lreu R	GCTTGTTGGTTTGGGCTCTTC			
Reuteransucrase (*gtfA*)	GTFA F	AATTAAACTGGTTATACTATCTC	160	57	28
	GTFA R	GAGTTCATACCATCTGCAGC			
Levansucrase (*ftfA*)	FTF F	TATCAATGATACAAATAATGC	1065	56	this study
	FTF R	GCGTTTCAGGATCATTTGGT			
	LEV F	GTCAATTTGATCCTTCGCC	460	57	this study
	LEV R	TGCAACTAAGGAAATTAAGGGC			
Non-ribosomal peptide synthase (*rtcN*)	RC F	GGCGGAACGTTGAATATTGT	248	60	this study
	RC R	ATTTTGGGGGAATCATAGCC			
Universal stress protein A	Ecoli F	CCGATACGCTGCCAATCAGT	884	66	55
	Ecoli R	ACGCAGACCGTAGGCCAGAT			
LT	LT F	CCGTGCTGACTCTAGACCCCCA	480	68	56
	LT R	CCTGCTAATCTGTAACCATCCTCTGC			
STa	STa F	ATGAAAAAGCTAATGTTGGC	193	65	57
	Sta R	TACAACAAAGTTCACAGCAG			
STb	STb F	TGCCTATGCATCTACACAAT	113	60	58
	STb R	CTCCAGCAGTACCATCTCTA			
K88 fimbriae	K88 F	GCACATGCCTGGATGACTGGTG	439	67	59
	K88 R	CGTCCGCAGAAGTAACCCCACCT			

[a] The *Lactobacillus* group includes *Lactobacillus* spp., *Pediococcus* spp., *Leuconostoc* spp. and *Weissella* spp.

[b] T_m, melting temperature.

Table 6.3. Concentration of metabolites in feed after 24 h of fermentation with *L. reuteri*[a]

L. reuteri strain (exopolysaccharide produced)	Amt of sucrose added (%)	Metabolite concn (mmol/kg feed)			Reuteran or levan concn (g/kg feed)[b]
		Lactate	Acetate	Ethanol	
TMW1.656 (reuteran)	10	87.1 ± 5.8	51.0 ± 4.2	36.2 ± 1.8	5.6 ± 1.0
	20	79.4 ± 0.1	71.3 ± 1.3	2.5 ± 0.0	3.7 ± 1.1
LTH5794 (levan)	10	80.7 ± 4.6	57.3 ± 3.7	27.31 ± 4.9	3.2 ± 0.6
	20	70.7 ± 3.3	67.1 ± 3.4	3.41 ± 4.8	3.7 ± 0.6

[a] Wheat four with addition of 10 or 20% sucrose was used as the substrate. The pH of unfermented wheat after inoculation was 6.1, and the pH after 24 h of fermentation was 3.7 for all fermentations.

[b] Reuteran and levan were quantified relative to the content of water-soluble polysaccharides in unfermented chemically acidified feed (34, 43).

Table 6.4. Growth performance of pigs fed supplemental fermented diet for 21 days

Parameter	ADFI (g DM[d]/day)	ADG (g/day)	G/F ratio
Dietary treatment[a]			
CTRL	305	255	0.76
ACID	295	264	0.84
TMW1.656			
REU^+	268	241	0.86
REU^-	283	240	0.79
LTH5794			
LEV^+	266	210	0.74
LEV^-	265	240	0.84
SEM	19	19	0.05
P value[b]			
Acids	0.659	0.712	0.231
Exopolysaccharides	0.644	0.403	0.628
L. reuteri + reuteran or levan	0.029[c]	0.046	0.894
L. reuteri	0.085	0.240	0.725
Fermentation	0.025	0.067	0.899

[a] Diets were supplemented with organic acid and fermented sourdough. Pigs were fed a phase 1 diet for the first 7 days, followed by a phase 2 diet from day 7 to 21.

Abbreviations for the diets are used as follows: CTRL, control; ACID, chemically acidified feed; REU^+ and REU^-, feed fermented with *L. reuteri* TMW1.656 with addition of sucrose to support reuteran formation (REU^+) or with addition of glucose and fructose (REU^-); LEV+ and LEV^-, feed fermented with *L. reuteri* LTH5794 with addition of sucrose to support levan formation (LEV^+) or with addition of glucose and fructose (LEV^-).

[b] For acid effects, *P* values are for the control diet versus chemically acidified diet; for reuteran or levan effects, *P* values are for sucrose-supplemented diets REU^+ and LEV^+ versus REU^- and LEV^-; for *L. reuteri* + reuteran or levan, *P* values are for reuteran- or levan-containing fermented diets (REU^+ and LEV^+) versus unfermented diets (CTRL and ACID); for *L. reuteri*, *P* values are for fermented diets without exopolysaccharides (REU^- and LEV^-) versus unfermented diets (CTRL and ACID); for fermentation, *P* values are for all fermented diets (REU^+, REU^-, LEV^+, LEV^-) versus unfermented diets (CRTL and ACID).

[c] *P* values of less than 0.05 are indicated in boldface.

[d] DM, dry matter.

Table 6.5. Gene copy numbers for the *Lactobacillus* group, *L. reuteri*, *gtfA, rtcN,* and *ftfA* obtained on days 0, 7, 14 and 21[a]

Organism or gene and time (day)	Log(gene copy no./g) for the following diet[b]					
	Control	Chemically acidified	TMW1.656 with sucrose	TMW1.656 with Glu + Fru	LTH5794 with sucrose	LTH5794 with Glu + Fru
Lactobacillus group						
0	$9.13 \pm 0.17^{A,X}$	$8.93 \pm 0.17^{A,X}$	$8.86 \pm 0.21^{A,X}$	$8.92 \pm 0.29^{A,X}$	$9.40 \pm 0.19^{A,X}$	$8.69 \pm 0.19^{A,B,X}$
7	$9.35 \pm 0.17^{A,B,C,X}$	$9.69 \pm 0.17^{A,Y}$	$9.02 \pm 0.17^{C,X}$	$9.51 \pm 0.17^{A,B,X}$	$9.32 \pm 0.17^{A,B,X}$	$9.10 \pm 0.17^{B,C,X,Y}$
14	$8.99 \pm 0.17^{A,X}$	$9.30 \pm 0.17^{A,B,X,Y}$	$9.32 \pm 0.17^{A,B,X}$	$9.48 \pm 0.17^{B,X}$	$9.05 \pm 0.17^{A,B,X}$	$9.06 \pm 0.17^{A,B,X,Y}$
21	$9.02 \pm 0.17^{A,X}$	$9.35 \pm 0.17^{A,X,Y}$	$9.23 \pm 0.17^{A,X}$	$9.21 \pm 0.17^{A,X}$	$9.22 \pm 0.17^{A,X}$	$9.17 \pm 0.17^{A,Y}$
L. reuteri						
0	$7.85 \pm 0.28^{A,X}$	$7.47 \pm 0.28^{A,B,X}$	$6.87 \pm 0.33^{B,X}$	$7.44 \pm 0.44^{A,B,X}$	$8.14 \pm 0.30^{A,X}$	$7.48 \pm 0.3^{A,B,X}$
7	$8.68 \pm 0.27^{A,Y,Z}$	$8.93 \pm 0.27^{A,Y}$	$8.25 \pm 0.27^{A,Y}$	$8.42 \pm 0.27^{A,Y}$	$8.81 \pm 0.27^{A,Y}$	$8.84 \pm 0.27^{A,Y}$
14	$8.98 \pm 0.19^{A,Z}$	$8.94 \pm 0.19^{A,Y}$	$8.17 \pm 0.19^{B,Y}$	$8.39 \pm 0.19^{B,X,Y}$	$8.59 \pm 0.19^{A,B,X,Y}$	$8.70 \pm 0.19^{A,B,Y}$
21	$8.38 \pm 0.16^{A,B,X,Y}$	$8.83 \pm 0.16^{B,Y}$	$7.44 \pm 0.16^{C,X}$	$8.05 \pm 0.16^{A,X,Y}$	$8.03 \pm 0.16^{A,X}$	$8.13 \pm 0.16^{A,X}$
Reuteransucrase (*gtfA*)						
0	<5	<5	< 5	< 5	< 5	< 5
7	<5	5.65 ± 0.26^{A}	5.93 ± 0.26^{A}	6.41 ± 0.26^{B}	< 5	< 5
14	<5	5.36 ± 0.26^{A}	6.42 ± 0.26^{B}	6.45 ± 0.26^{B}	< 5	< 5
21	<5	5.65 ± 0.26^{A}	5.95 ± 0.26^{A}	6.80 ± 0.26^{B}	< 5	< 5
Levansucrase (*ftfA*)						
0	6.67 ± 0.30	<6	< 6	< 6	< 6	< 6
7	6.08 ± 0.17^{A}	<6	< 6	< 6	8.36 ± 0.17^{B}	8.23 ± 0.17^{B}

14	6.90 ± 0.13^{A}	6.72 ± 0.13^{A}	6.79 ± 0.13^{A}	6.69 ± 0.13^{A}	7.82 ± 0.13^{B}	8.26 ± 0.13^{C}
21	6.22 ± 0.20^{A}	<6	< 6	< 6	8.09 ± 0.20^{B}	8.38 ± 0.20^{B}
Nonribosomal peptide synthase (*rtcN*)						
0	<6	<6	< 6	< 6	< 6	< 6
7	<6	<6	6.81 ± 0.29	7.17 ± 0.29	< 6	< 6
14	<6	6.46 ± 0.29	6.57 ± 0.34	6.92 ± 0.34	< 6	< 6
21	<6	<6	6.24 ± 0.25	6.86 ± 0.25	< 6	< 6

[a] *gtfA* is a marker for *L. reuteri* TMW1.656 in feces, *rtcN* is a second marker for *L. reuteri* TMW1.656 in feces, and *ftfA* is a marker for *L. reuteri* LTH5794 in feces. Data are presented as least-square means (n = 36) ± standard error of the means. Superscripts A, B and C denote significant differences ($P < 0.05$) between diets at each time point (comparison across rows); superscripts X, Y and Z denote significant differences ($P < 0.05$) within a diet over time (comparison across columns). Values that do not share a common superscript are significantly different. The detection limit for *gtfA* was 5 $\log_{10}$ gene copies/g of feces (wet weight); the detection limit for the *Lactobacillus* group, *L. reuteri*, *rtcN* and *ftfA* was 6 $\log_{10}$ gene copies/g of feces (wet weight).

[b] For TMW1.656 and LTH5794, the diets consisted of feed fermented with TMW1.656 and LTH5794, respectively, and supplemented with the indicated sugar.

Table 6.6. Gene copy number for *E. coli*, STb, LT, K88 fimbriae, and STa in feces obtained on days 0, 7, 14 and 21[a]

Bacterium or bacterial toxin and time (day)	Log(gene copy no./g) for the following diet[b]					
	Control	Chemically acidified	TMW1.656 with sucrose	TMW1.656 with Glu + Fru	LTH5794 with sucrose	LTH5794 with Glu + Fru
E. coli						
0	$6.66 \pm 0.45^{A,X}$	$6.70 \pm 0.45^{A,X}$	$6.91 \pm 0.55^{A,X,Y}$	$6.74 \pm 0.78^{A,X}$	$7.72 \pm 0.49^{A,X}$	$7.09 \pm 0.49^{A,X}$
7	$6.86 \pm 0.45^{A,B,X}$	$6.61 \pm 0.45^{A,X}$	$7.98 \pm 0.45^{B,X}$	$6.61 \pm 0.45^{A,X}$	$7.74 \pm 0.45^{A,B,X}$	$7.59 \pm 0.45^{A,B,X}$
14	$7.13 \pm 0.45^{A,X}$	$6.95 \pm 0.45^{A,X}$	$6.17 \pm 0.45^{A,Y}$	$6.51 \pm 0.45^{A,X}$	$7.29 \pm 0.45^{A,X}$	$7.02 \pm 0.45^{A,X}$
21	$6.42 \pm 0.45^{A,X}$	$5.96 \pm 0.45^{A,B,X}$	$4.77 \pm 0.45^{B,Z}$	$5.36 \pm 0.45^{A,B,X}$	$5.22 \pm 0.45^{A,B,Y}$	$5.21 \pm 0.45^{A,B,Y}$
STb						
0	< 4	< 4	< 4	< 4	< 4	< 4
7	$5.07 \pm 0.82^{A,X}$	$5.83 \pm 0.82^{A,X}$	$7.84 \pm 0.82^{B,X}$	$6.10 \pm 0.82^{A,B,X}$	$6.78 \pm 0.82^{A,B,X,Y}$	$6.79 \pm 0.82^{A,B,X,Y}$
14	$7.76 \pm 0.76^{A,Y}$	$7.70 \pm 0.76^{A,B,X}$	$6.51 \pm 0.76^{A,X}$	$6.29 \pm 0.76^{A,X}$	$8.05 \pm 0.76^{A,Y}$	$7.03 \pm 0.76^{A,Y}$
21	$6.94 \pm 0.69^{A,X}$	$6.51 \pm 0.69^{A,B,X}$	$4.27 \pm 0.69^{C,Y}$	$5.04 \pm 0.69^{A,B,C,X}$	$4.91 \pm 0.69^{B,C,X}$	$4.69 \pm 0.69^{B,C,X}$
LT						
0	< 4	4.29 ± 0.26^{X}	4.24 ± 0.33^{X}	< 4	4.23 ± 0.29^{X}	4.84 ± 0.29^{X}
7	6.16 ± 0.73^{X}	5.73 ± 0.73^{Y}	6.56 ± 0.73^{Y}	6.22 ± 0.73^{X}	6.19 ± 0.73^{Y}	5.78 ± 0.73^{X}
14	7.29 ± 0.75^{X}	7.19 ± 0.75^{Y}	6.11 ± 0.75^{Y}	6.68 ± 0.75^{X}	6.91 ± 0.75^{Y}	6.34 ± 0.75^{X}
21	4.97 ± 0.33^{Y}	4.10 ± 0.33^{X}	< 4	4.08 ± 0.33^{Y}	4.44 ± 0.33^{X}	4.85 ± 0.33^{X}
K88 fimbriae						
0	< 4	< 4	< 4	< 4	< 4	< 4
7	$5.43 \pm 0.74^{X,Y}$	4.86 ± 0.74^{X}	5.82 ± 0.74^{Y}	5.59 ± 0.74^{X}	5.63 ± 0.74^{X}	5.15 ± 0.74^{X}

14	6.53 ± 0.82^{X}	6.39 ± 0.82^{X}	5.29 ± 0.82^{X}	5.90 ± 0.82^{X}	6.15 ± 0.82^{X}	5.31 ± 0.82^{X}
21	4.32 ± 0.42^{Y}	< 4	< 4	< 4	< 4	4.15 ± 0.42^{X}
STa						
0	<4	<4	<4	<4	<4	4.11 ± 0.16
7	<4	<4	<4	<4	<4	<4
14	<4	<4	<4	4.13 ± 0.16	<4	<4
21	<4	<4	<4	<4	<4	<4

[a] Data are presented as least-square means (n = 36) ± standard error of the means. Superscripts A, B and C denote significant differences ($P < 0.05$) between diets at each time point (comparison across rows); superscripts X, Y and Z denote significant differences ($P < 0.05$) within a diet over time (comparison across columns). Values that do not share a common superscript are significantly different. The detection limit for *E. coli,* STb, LT, K88 fimbriae, and STa was 4 $\log_{10}$ gene copies/g of feces (wet weight).

[b] For TMW1.656 and LTH5794, the diets consisted of feed fermented with TMW1.656 and LTH5794, respectively, and supplemented with the indicated sugar.

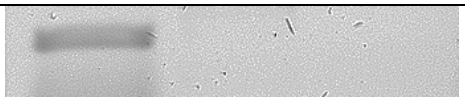
460 bp: (T_m 82.1°C)	 Neg. Control TMW1.656 LTH5794	*ftfA* (*L. reuteri* LTH5794)
160 bp (T_m 77.5°C)	 TMW1.656 LTH5794 Neg. Control	*gtfA* (*L. reuteri* TMW1.656)
250 bp (T_m 77.5°C)	 TMW1.656 LTH5794 Neg. Control	*rtcN* (*L. reuteri* TMW1.656)

Figure 6.1. Validation of specificity of primers for strain-specific PCR detection of *L. reuteri* TMW1.656 and LTH5794. Specific amplification was verified by determination of the uniform and matching melting temperatures (T_ms) in qPCRs and by determination of the sizes of the amplicons.

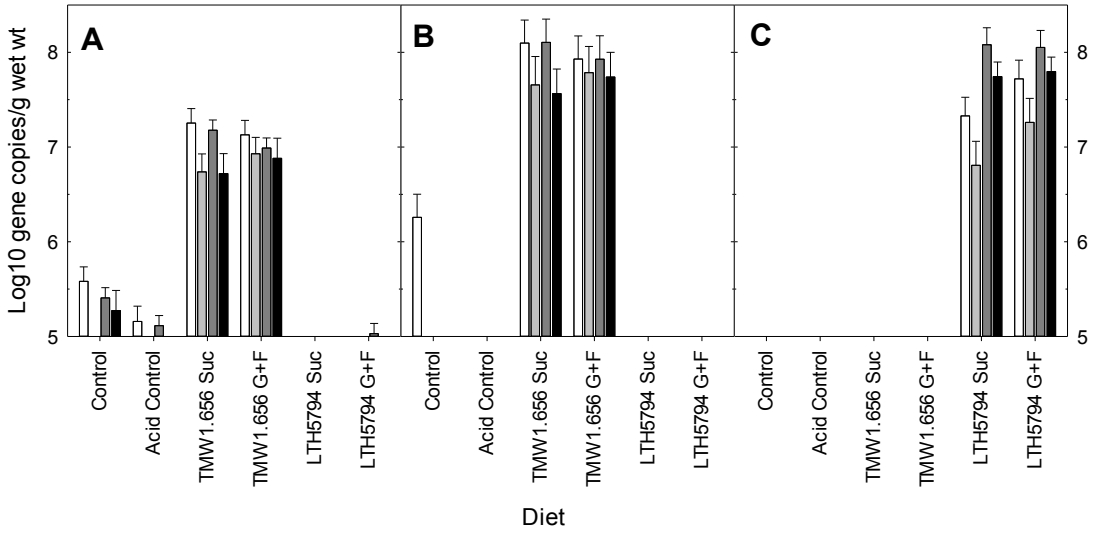


Figure 6.2. Copy numbers of the genes *gtfA* as marker for *L. reuteri* TMW1.656 (A); *rtcN* as a second marker for *L. reuteri* TMW1.656 (B), and *ftfA* as a marker for *L. reuteri* LTH5794 (C) in digesta obtained from the stomach (white bars), ileum (light gray bars), cecum (dark gray), or colon (black). Data are presented as least-square means (n = 36), with their standard errors being represented by vertical bars. The copy numbers of the *gtfA* gene were significantly ($P < 0.05$) higher in samples from animals fed *L. reuteri* TMW1.656 than in samples from all other animals. The copy numbers of the *rtcN* gene were significantly ($P < 0.05$) higher in samples from animals fed *L. reuteri* TMW1.656 than in samples from all other animals. The detection limits for Lactobacillus group, *L. reuteri* and levansucrase were 6 $\log_{10}$ gene copies/g of digesta (wet weight). Acid Control, chemically acidified; Suc, sucrose; G, glucose; F, fructose.

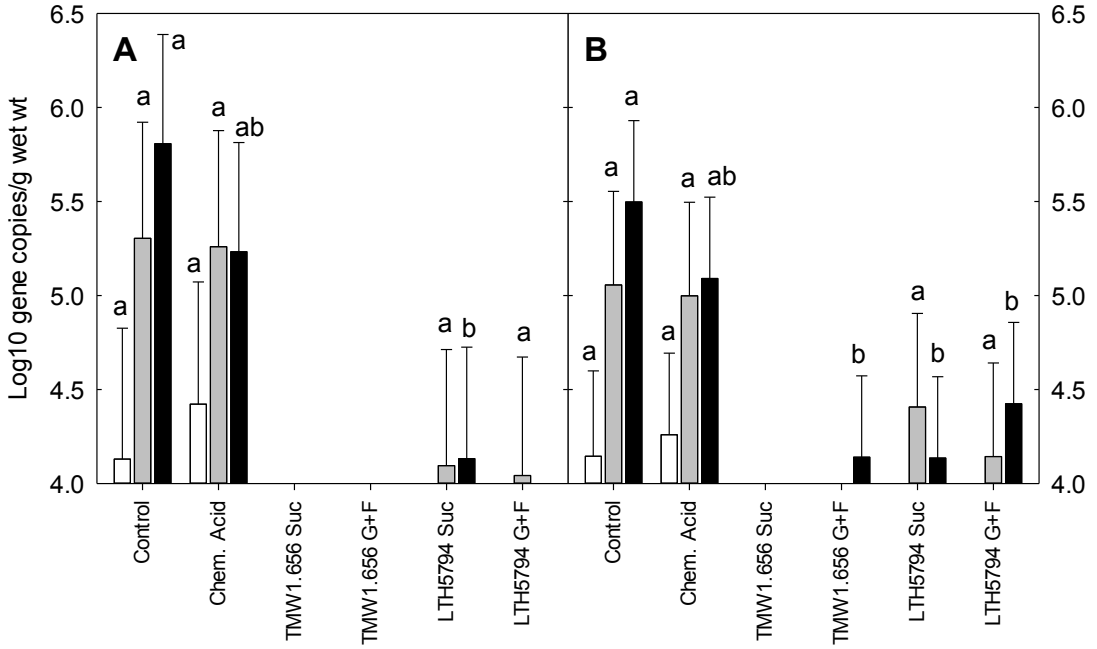


Figure 6.3. Gene copy numbers for *E. coli* (A) and its STb virulence factor (B) in the gut digesta obtained from the ileum (white bars), cecum (light gray bars), or colon (black bars). Data are presented as least-square means (n = 36), with their standard errors being represented by vertical bars. Superscripts A, B and C denote significant differences ($P < 0.05$) between diets at each site. Values not having the same letter are significantly different. The detection limit was 4 $\log_{10}$ gene copies/g of digesta (wet weight). Chem. Acid., chemically acidified; Suc, sucrose; G, glucose; F, fructose.