Feed fermentation formulate gut microbiota of weanling pigs

- Effects of exopolysaccharides and reutericyclin

by

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Abstract

Enteric bacterial infections, especially post-weaning diarrhea, are among the most common diseases affecting pigs and economically significant in swine production. Antibiotics were a cost-effective method to treat and prevent these bacterial diseases. However, with concerns of the prevalence of antimicrobial resistance bacteria from extensive usage, antibiotics are limited or prohibited to be used for animal growth promoter in many countries. Great interest of research in alternatives was thus raised. Lactic acid bacteria (LAB) and their metabolites were found to be effective in protecting against *E. coli* infection both *in vitro* and *in vivo*. This thesis aims to evaluate the potential application of exopolysaccharides synthesized by LAB to prevent and treat post-weaning diarrhea and its effects on pigs' fecal microbiota in a pig feeding trial.

Pigs (36) were divided into 6 treatment groups and fed wheat, acidified wheat, or wheat fermented with *Lactobacillus reuteri*. Fecal samples were obtained at weaning and 1, 2, or 3 weeks after weaning and gut digesta samples were collected from week 3 of weaning when the pigs were euthanized. Quantitative PCR results indicated the decreasing gene counts of *E. coli* and its virulence factors in pigs fed with fermented wheat. Reuteran containing diets had a better performance than other diets and have the potential in preventing and controlling *E. coli* infections in pigs. Diets supplemented with *L. reuteri* fermented feed have a promising future in the swine industry.

Microbiota analysis was based on sequencing of 16S rRNA gene amplicons; sequences were evaluated with the QIIME pipeline. Sequencing provided more than 7939 sequences per sample. The composition of microbiota changed over time ($p < 0.0001$) and the diversity of microbiota increased at week 3 ($p < 0.001$). At week 3, microbiota were composed of *Bacteroidetes* and *Firmicutes*, accounting for up to 90% of sequences, followed by *Tenericutes, Spirochaetes, Proteobacteria, Planctomycetes*, and unassigned organisms. *Tenericutes* increased over time; *Proteobacteria* peaked at week 1 but decreased thereafter. Changes within the *Bacteriodetes* and *Firmicutes* were at the family level; the overall increase of microbial diversity was mainly attributable to an increase of unassigned organisms and several genera in the phylum *Firmicutes*. When compared to the effect of time, only few significant changes of intestinal microbiota were attributable to the diet. In conclusion, this is the first in depth analysis of the development of piglet microbiota after weaning. Results will support future efforts to manage the transition period in commercial pig production.

Preface

This thesis was written based on the guidelines for a paper format thesis of the Faculty of Graduate Studies and Research at the University of Alberta.

The animal study was conducted according to the guidelines of the Canadian Council on Animal Care and was approved by the Animal Care and Use Committee: Livestock of the University of Alberta, Edmonton, AB, Canada. The feeding trial was done by Sandra Galle, Hong Anh Le Minh and myself.

Chapter 1 is a literature review and it is my original work. The relevant information about enteric bacterial diseases of swine, usage of antibiotics in swine industry and the proposed alternatives of antibiotics were assembled in this review.

Chapter 2 is in preparation for submission as: Yan Yang, Hong Anh Le Minh, Sandra Galle, Ruurd T. Zijlstra, Michael G. Gänzle. "Exopolysaccharide synthesized by *Lactobacillus reuteri* in fermented feed have positive effects on protecting against *E. coli* in weaning pigs." The microbial analysis by quantitative PCR (qPCR) and the data analysis as well as the manuscript were my original work. Sandra Galle wrote the methods of feed fermentation and preparation. Hong Anh Le Minh analyzed the growth performance data.

Chapter 3 is prepared for submission as: Yan Yang, Hong Anh Le Minh, Ruurd T. Zijlstra, Michael G. Gänzle. "Effects of reutericyclin on fecal microbiota of weanling pigs." DNA extraction, Illumina sequences analysis, qPCR data analysis and the manuscript were my contribution. qPCR were ran by Xin Zhao.

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III. List of Abbreviations

DNA	Deoxyribonucleic acid
PIS	Porcine intestinal spirochaetosis
PCS	Porcine colonic spirochaetosis
PE	Proliferative enteropathy
NAHMS	National Animal Health Monitoring System
AHI	Animal Health Institute
ADG	Average daily weight gain
ADFI	Average daily feed intake
G:F	Feed efficiency (ADG/ADFI)
BLAST	Basic Local Alignment Search Tool
QIIME	Quantitative Insights Into Microbial Ecology
NRC	National Research Council Canada
TiO_2	Titanium dioxide
OTU	Operational taxonomic units
RDP	Ribosomal database project
PD	Phylogenetic diversity
SAS	Statistical analysis system
PCoA	Principle coordinate analysis
Bp	Base pair
LAB	Lactic acid bacteria
qPCR	Quantitative polymerase chain reaction

1. Common enteric bacterial diseases of swine: a literature review

1.1 Life cycle of the pig

According to the code of practice for the care and handling of pigs in Canada (Canada, 2014), there are five stages of raising pigs for meat production: Gestation (pregnancy of the sow), farrowing (giving birth to the piglets), nursery (suckling and weaning of young piglets), growing and/or finishing (growth of weaned piglets to slaughter weight). There is no strict definition of age and weight in each stage.

Beginning with breeding of a sow, the gestation phase is 115 days. Sows normally have two litters each year with an average of 10 to 12 piglets per litter. The piglets are allowed to suckle for 3 to 4 weeks, then they are moved to a nursery for 4 to 8 weeks. Then the growing-finishing stage begins, where piglets are divided into equal groups and fed high energy feed. The pigs are slaughtered when they reach market weight, approximately 110 kg. The time to raise a pig from farrow to finish is approximately 5 to 6 months. Some producers only raise piglets from farrow to nursery while others complete the finishing stage; however, most producers raise pigs from farrow to finish.

1.2 Pigs gut microbiota

The porcine gut microbial ecosystem are complex and dynamic, the composition differs between pig ages, gastrointestinal location and individuals (Konstantinov et al., 2004b). The gut is colonised by microbes including lactic acid bacteria, enterobacteria and streptococci from the mother and the environment after birth (Lallès et al., 2007). The number and diversity of obligate anaerobes increased after weaning because of

the transition from liquid milk to solid feed (Inoue et al., 2005; Konstantinov et al., 2006). Mature pigs have a quite stable microbiota in the gut tract. The gastrointestinal microbiota of pigs is primarily consisted of Gram-positive bacteria like *Streptococcus*, *Lactobacillus*, *Bifidobacterium*, *Peptostreptococcus*, *Clostridium*, *Ruminococococcus* and *Escherichia*, and Gram-negative bacteria such as *Bacteroides*, *Fusobacterium*, *Prevotella* and *Butyrivibrio* (Gaskins, 2001).

There are mutual interactions between pigs and gastrointestinal microbiota. On one hand, factors including host genotype and environmental factors like milk-associated microbiota and diet after weaning have strongly effects on microbiota development (Bauer et al., 2006). While *L. sobrius (L. amylovorus)*, *L. reuteri* and *L. acidophilus* are the major components of pig microbiota before weaning, their numbers decreased significantly after weaning (Konstantinov et al., 2006). On the other hand, pigs are supported by the gut microbiota, which can compete with pathogens with nutrients and adhesion sites or produce antimicrobial compounds as well as modulate the immune reaction (Rolfe, 1997; Fanning et al., 2012). Unlike adults, the neonatal and weaning piglets are highly susceptible to enteric diseases (Hopwood & Hampson, 2003). In the immediate post-weaning period, there are remarkable shifts in the composition and metabolic activities of microbiota (Konstantinov et al., 2006). Several potentially beneficial bacteria are suppressed during this period. The development of so-called healthy commensal microbiota can be easily broken and the bacterial intestinal disease can be established (Hopwood & Hampson, 2003). Hence,

the study of gut microbiota, especially during early weaning period is of special importance in swine production.

1.3 Major enteric bacterial diseases of pigs

Enteric bacterial infections are among the most common diseases pigs and they are economically significant in swine production around the world. The clinical signs of these diseases normally include: diarrhea, weight loss, decreased growth rate and even death of neonatal, weaning, and/or growing-finishing pigs. *Clostridium perfringens*, *Clostridium difficile, Escherichia coli*, *Salmonella enterica*, *Brachyspira hyodysenteriae, Brachyspira pilosicoli* and *Lawsonia intracellularis* are the most common etiological agents of these infections (Table 1-1). This review gives a brief introduction of these enteric diseases and their impact on pigs.

Table 1-1. Enteric diseases at various ages of pigs.

Infected agent	Newborn	Suckling (2-5kg)	Weaning (5-20kg)	Grower (20-70kg)	Finisher (>70kg)	Adults
Enterotoxigenic *E. coli*	++++	++++	++++	+	-	-
C. difficile	++++	++	-	-	-	-
C. perfringens Type A	++++	++	-	-	-	-
C. perfringens Type C	++++	++	+	-	-	-
S. enterica	-	+	++	++++	-	+
B. hyodysenteriae	-	+	++	++++	++++	++
B. pilosicoli	-	-	++	++++	++++	++
L. intracellularis	-	-	++	++++	++++	++

Note: '-' indicates pigs are seldom, if ever, infected; 1+, 2+, and 3+ indicate increasing frequencies of infection, with 4+ indicates the age groups that are most

often infected. The division of the pig stages is based on the code of practice for the care and handling of pigs in Canada (Canada, 2014).

1.3.1 Clostridial enteric infections

C. difficile and *C. perfingens* are the principal etiology agents of clostridial enteric infections in swine. *C. difficile* is a major cause of antibiotic-associated diarrhea in neonatal pigs at 1 to 7 days of age (Songer et al., 2000). Infected pigs experienced early-onset scours, sometimes with respiratory distress and sudden death (Songer & Uzal, 2005). Monomeric enterotoxin A (308kDa) and cytotoxin B (270kDa) produced by *C. difficile* are responsible for the systemic effects (Songer & Uzal, 2005; Steele et al., 2010). Type A and C toxins of *C. perfingens* are the most common cause of clostridial enteric diseases. Type A, is a member of normal intestinal microbiota of swine (Månsson and Smith, 1962), but it can cause enteric disease in neonatal pigs during the first week of life (Jestin et al., 1985). Beta2 toxin was suggested to play an important role in type A infection and can be produced by nearly all type A strains (Bueschel et al., 2003; Waters et al., 2003). Clinical signs include: mucosal necrosis, villus atrophy and serositis, with lesions being most severe in the jejunum and ileum (Popoff & Jestin, 1985). The diagnosis of type A infections is complicated by the difficulties in differentiating the normal microbiota and infectious strains. Type C infection generally occurs in piglets less than 7 days of age but it has been documented as early as 12 hours after birth to 3-day-old piglets (Matthias et al., 1965). This disease is more severe than type A infection and is characterized by fatal

hemorrhagic and necrotic enteritis (Songer & Meer, 1996). The key factor mediating type C infections is the lethal and necrotizing beta toxin (CPB) (Hogh, 1967a).

In swine production, the prevalence of *C. difficile* infection can be as high as 90% with an average of 47.6% on a per litter basis (Songer & Uzal, 2005). The infections of *C. perfringens* type A in pigs is unpredictable because all strains of this type can cause disease under the right conditions (Songer & Uzal, 2005). Prevalence of *C. perfringens* type C can be 100% in infected litters, with mortality rate as high as 50-60% (Hogh, 1967b).

1.3.2 *E. coli* infections

E. coli is the most significant cause of diarrhea and edema diseases (ED) in neonatal and post-weaning pigs, which will be discussed in more detail below. The diseases include neonatal diarrhea, post-weaning diarrhea (PWD) and ED. *E. coli* associated neonatal diarrhea often occurs in pigs less than 4 days old. PWD and ED commonly occur in weaning pigs. They may occur independently or together in a single outbreak but there are important differences between the two diseases. Compared with infections in newborn piglets, post-weaning infections are more common and severe. The details of PWD are discussed in the following paragraphs.

Post-weaning colibacillosis

Post-weaning colibacillosis (PWC), also known as PWD, is a disease mainly caused by enterotoxigenic *Escherichia coli* (ETEC) in the small intestine (Moon et al., 1977; Fairbrother et al., 2005; Duan et al., 2013). ETEC commonly infects neonatal and

weanling pigs with ages of less than one day to approximately eight weeks (Moxley & Duhamel, 1999). Watery diarrhea is the common symptom and the death of the pig generally results from dehydration.

ETEC strains possess proteinaceous appendages or specific fimbriae for intestinal attachment and colonization (Parry & Rooke, 1985), which is the first step for establishing the enteric infection (Nagy & Fekete, 2005). After the initial colonization, ETEC produce proteins, enterotoxins, which induce severe watery diarrhea by increasing secretion and reducing absorption of electrolytes and water in the intestine (Gyles, 1994; Blanco et al., 1997). In addition to adhesions and enterotoxins, which are two main virulence factors of ETEC, receptors present in the gut also play an important role in pathogenesis (Nagy & Fekete, 2005). Thus, due to specificities of the receptor, ETEC infections seem to be host specific, so that pig ETEC strains might not cause problems for man or other animals.

ETEC that cause diarrhea in swine are associated with five types of fimbriae: F4 (K88), F5 (K99), F6 (987P), F7 (F41) and F18 (Ørskov & Ørskov, 1983; Wilson & Francis, 1986; Nagy & Fekete, 1999). Diarrheal diseases in pigs are most frequently caused by F4 (K88) and F18 fimbriae (Melkebeek et al., 2012). According to Frydendahl (2002), in a total of 219 isolates, 96 and 86 isolates were identified as F4 and F18 fimbriae, respectively. F4 are mainly found in ETEC infections of newborn and suckling pigs, while F18 are associated with PWD (Fairbrother et al., 2005). F4ab, ac and ad are antigenic variants of F4 fimbriae (Ørskov et al., 1964; Guinée & Jansen,

1979) with F4ac being the most common (Fairbrother et al., 2005). F18 fimbriae occur as two antigenic variants, F18ab and F18ac (Rippinger et al., 1995). In addition to the five traditional fimbrial antigens mentioned above, another virulence factor, Adhesin Involved in Diffuse Adherence (AIDA) has been described (Ngeleka et al., 2003). AIDA was reported to play an important role in PWD caused by ETEC in pigs (Ha et al., 2003).

Heat stable toxin (ST) and heat labile (LT) toxin, first described by Smith and Gyles (1970), are two important enterotoxins produced by ETEC strains that induce severe watery diarrhea in pigs. There are two types of ST enterotoxins: STa, which is soluble in methanol and active in newborn pigs; and STb, which is methanol insoluble and can induce fluid secretion in both weaned and newborn pigs (Burgess et al., 1978; Whipp, 1991; Nagy & Fekete, 1999b). LT enterotoxins, found by Gyles and Barnum (1969) and were derived from cholera toxin (CT) of *Vibrio cholerae*, are mainly produced by human and porcine ETEC strains (den Akker et al., 1996; Hirst et al., 1998). LT also contains two major serogroups: LT-I and -II. LT-I is expressed by *E. coli* strains in both human and animal infections, while LT-II is found predominately in *E. coli* isolates from animals (Nataro & Kaper, 1998). Another enterotoxin heat stable enterotoxin 1 (EAST1) is highly prevalent. The EAST1 gene was detected in porcine ETEC strains, but its role in ETEC-induced diarrhea is not clear (Savarino et al., 1996; Choi et al., 2001; Zhang et al., 2006).

Generally, ETEC infections in pigs begin with the ingestion ETEC strains from food or water. When the strains colonize the small intestine by attaching to receptors present on the epithelium using their specific fimbrial adhesins. They then proliferate to approximately 10^9 CFU per gram of tissue in the mid-jejunum to the ileum. Whether the infections result in disease or not depends on the degree of colonization. After colonization, the bacteria produce enterotoxins. LT elevates the intracellular level of cyclic adenosine monophosphate (cAMP), causing activation of the chloride channel and secretion of chloride from the apical region of enterocytes (Thiagarajah & Verkman, 2003). Haan and Hirst (2004) reviewed the relationship between cAMP accumulation and chloride secretion causing diarrhea. In contrast, STa functions by increasing levels of cyclic guanosine monophosphate (cGMP) in enterocytes; however, both toxins induce diarrhea by promoting the secretion of chlorides (Forte et al., 1992). The increased secretion of chloride and inhibition of sodium absorption results in a massive loss of water moving into the intestinal lumen and severe secretory diarrhea. The mechanism of STb-induced diarrhea is not well understood. STb is reported to bind different receptors; however, it does not stimulate production of cyclic nucleotides (Weikel et al., 1986; Harville & Dreyfus, 1995).

The prevalence of *E. coli*-induced diarrhea and losses associated with PWD have been reported worldwide. In a study conducted in Ontario in 1999, researchers reported that the estimated annual financial loss caused by PWD is $20,000 in a 500-head sow herd, with mortality less than 5% (Amezcua et al., 2002). However, the morbidity in ETEC-

affected herds may be up to 80% in a particular litter with an average of 30-40% (Fairbrother et al., 2005). In North Vietnam, ETEC was responsible for 43% of cases of diarrhea in piglets 4 days old and 23.9% of the remaining cases at weaning (Do et al., 2006). In Korea between 1995 and 1998, 853 *E. coli* isolates from 957 pre-weaning diarrheic pigs (less than 14 days old) and 250 *E. coli* isolates from 294 weaning pigs with diarrhea were reported (Choi et al., 2002).

1.3.3 Swine Dysentery

Swine dysentery (SD) is a mucohaemorrhagic colitis in grower and finisher pigs caused by the anaerobic spirochaete, *Brachyspira hyodysenteriae* (Harris, 1999). This etiological agent mainly affects the cecum, colon and rectum and induces extensive inflammation and erosion of the epithelial surface of the gut (Hampson, 2006). In typical cases of SD, the clinical signs of infected pigs are reduced feed intake, depression of growth rate and soft or porridge-like diarrhea (Pluske et al., 2002). After developing clinical signs, about 10% of affected pigs die within 5 days if they are left untreated (Hampson, 1995).

The precise pathogenesis of SD is still unclear. Despite the presence of the pathogen, the disease does not always induce clinical symptoms (Hampson et al., 1992). In a study investigating the etiology of SD, researchers found no clinical signs of disease after oral inoculation of spirochetes (*B. hyodysenteriae*) with or without *Vibrio coli*, (Meyer et al., 1974). A serological herd survey from Western Australia showed

similar findings (Mhoma et al., 1992). *B. hyodysenteriae* was detected in 33% of herds; however, little clinical disease was observed.

SD is a worldwide disease of pigs that causes significant problems in Europe. In a recent survey, researchers collected 1176 individual samples from 95 herds from Southern Germany and found the overall prevalence of *B. hyodysenteriae* was 8.4% in fecal samples and 24.2% in herds (Reiner et al., 2011). The presence of *B. hyodysenteriae* was significantly associated with severity of diarrhea (Reiner et al., 2011). In Poland, *B. hyodysenteriae* was detected in fecal samples from 8 of 23 herds (34.8%) (Plawinska et al., 2004). A 1996 postal survey in the U.K. indicated that 50.5% of 105 pig units had experienced problems with scours in the previous three years, 10.5% of which were identified as SD (Pearce, 1999). In Brazil it was reported that rectal swabs from 6 of 17 (35.3%) farms with a history of diarrhea were positive for *B. hyodysenteriae* (Barcellos et al., 2000). Information about the prevalence in the other parts of world is limited.

1.3.4 Porcine intestinal spirochetosis /colonic spirochetosis

Porcine intestinal spirochetosis (PIS), also known as Colonic Spirochetosis (PCS) or spirochetal diarrhea, is a spirochete-associated colitis caused by *Brachyspira pilosicoli* in weaner and grower pigs (Trott et al., 1996). The infection caused by the hemolytic, anaerobic intestinal spirochete is distinct from *B. hyodysenteriae* (Zuerner et al., 2004). Clinically, infected pigs lose body condition and excrete loose and sticky feces. This infection normally does not lead to the death of pigs. The disease may lead

to reduced growth rate and increased time to market weight, resulting in economic loss (Duhamel et al., 1998). However, the overall financial loss caused by PIS has not been critically evaluated.

PIS is distributed in pig herds throughout the world. In Germany, the prevalence of *B. pilosicoli* was 3.2% in 1176 fecal samples and 31.6% in 95 herds (Reiner et al., 2011). In Denmark, *B. pilosicoli* was found in 15 of 79 (19%) randomly selected herds, with 5-10% within-herd prevalence (Stege et al., 2000). *B. pilosicoli* was obtained from 6 of 7 (85.6%) herds showing signs of diarrhea in Sweden and from only 1 of 8 herds without diarrhea (Fellstrom et al., 1996), whereas it was isolated from 14 of 50 (28%) healthy farms in Finland (Heinonen et al., 2000).

1.3.5 Salmonellosis

There are two main concerns related to *Salmonella* infections in the swine industry: (1) salmonellosis disease of the animals; (2) contamination of pork products when pigs are infected with *Salmonella* spp. (Pluske et al., 2002). When infected by *salmonella*, the clinical symptoms of pigs normally include: loss of appetite, a febrile response with dullness and watery diarrhea (Griffith, 2006). *Salmonella* Typhimurium is the serotype that is most commonly associated with salmonellosis in pigs (van der Wolf et al., 1999). It has been reported that the infection of serovar Typhimurium resulted in severe clinical signs and even sudden death of pigs (Letellier et al., 1999). Growing-finishing pigs are the most frequently affected groups. Studies have shown that strains

persist in various tissue for many days (Cote et al., 2004). Thus, the presence of those strains in growing-finishing pigs also induces a food safety concern.

Salmonellosis occurs worldwide. In The Netherlands, *Salmonella* spp. were recovered from 71 of 306 herds (23%) (van der Wolf et al., 1999). An investigation in Germany showed that 28.3% of the 60 fattening, 50.0% of the 20 breeding and 15.0% of 20 farrow-to-feeder herds were serologically positive for *Salmonella* (Von Altrock et al., 2000). Salmonellae were isolated from 48% of fatal cases of diarrhea and septicemia and about 10% of scouring pigs in Taiwan (Hsu et al., 1983).

1.3.6 Proliferative Enteropathy

Proliferative enteropathy (PE) is a common pig disease caused by *Lawsonia intracellularis*, a Gram-negative, obligate intracellular bacterium (McOrist et al., 1995). PE, also known as proliferative ileitis, includes acute and chronic conditions, both characterized by mucosal thickening in the small intestine and colon (Pluske et al., 2002). A hyperplastic to adenoma-like mucosa was formed in affected tissues with a proliferation of crypts immature epithelial cells. The acute form mainly affects older pigs in which hemorrhagic diarrhea and sudden death are observed; whereas, the chronic form is observed in young growing pigs with signs of diarrhea, rough hair-coat and retarded growth (Bane et al., 2001). The infections are commonly found in pigs aged from 6 to 20 weeks with clinical signs usually appearing after moving or transport stress (Knittel, 1999). With subclinical infections, pigs only show poor growth performance.

PE is also prevalent all over the world. In Denmark, *L. intracellularis* was detected in 93.7% of herds but it was the only causative agent of infection in 32% of farms surveyed (Stege et al., 2000). All herds examined in Korea tested positive for serum antibodies of *L. intracellularis* infections (Lee et al., 2001). In Sweden, 285 samples from 50 herds, 47.6% of total 105 herds surveyed, were positive for *L. intracellularis* (Jacobson et al., 2005).

1.4 Antibiotic usage in swine industry

Since the discovery of penicillin in the 1940s (Waksman, 1947), antibiotics that kill or inhibit the growth of certain types of microorganisms and have been widely used in human and animal therapy (Thomke & Elwinger, 1998). In farm animals, antibiotics have been used for three major purposes: (1) as therapy to treat infection by identified bacteria, (2) as prophylactics to prevent outbreaks of bacterial diseases, and (3) as growth promoters to enhance performance (Sun et al., 2004). When used therapeutically, one or more veterinary antibiotics are used to treat an individual animal or a group of sick animals during certain periods (van den Bogaard & Stobberingh, 1999). Similarly, when used prophylactically, antibiotics are administered to prevent or control the spread of an existing bacterial infection in the herd. Antibiotics fed for growth promotion are normally used in low dosages. They modulate the bacterial population, microbial metabolism and metabolites in the gastrointestinal tract of animals to improve the efficiency of feed utilization and thus enhance growth performance (Droumev, 1983; Walton, 1983).

Antibiotics are of great importance in swine management. They are the most cost-effective way to improve growth and feed efficiency in both young pigs and growing-finishing pigs (Cromwell, 2002). All of the enteric diseases reviewed above were treated or prevented by use of antibiotics. In young pigs (weight from 7 to 25 kg) fed antibiotics, growth rate and feed efficiency were increased by 16.4% and 6.9%, respectively (Hays, 1977; Zimmerman, 1986). Antibiotics increased growth rate by 10.6% and feed efficiency by 4.5% in growing pigs (weight from 17 to 49kg) (Hays, 1977; Zimmerman, 1986). During the growing-finishing period (from 28 to 89kg), antibiotics improved growth rate and feed efficiency by 4.2% and 2.2%, respectively (Hays, 1977; Zimmerman, 1986). In addition to improving growth and feed efficiency, antibiotics are also administered to prevent and treat bacterial infections, especially in young pigs.

Due to promotion of growth and feed efficiency as well as disease prevention and treatment, a large proportion of pigs are fed with antibiotics in the swine industry. In the past three decades, antimicrobial agents have been used in more than 70% of pig starters, 70% of grower diets, 50% of finisher diets, and 40% of sow feeds (Cromwell, 2002). A national swine survey, conducted by National Animal Health Monitoring System (NAHMS) and the National Agricultural Statistics Service in 2006, indicated that 85.3% of nursery sites and 81.2% of grower/finisher sites used antibiotics in feed and over 50% of the nursery and grower/finisher pigs in these sites were given antibiotics (Swine, 2006). In a 2012 report, antibiotics were added to weaned pig diets

in nearly 40% of operations they monitored (Swine, 2012). The Animal Health Institute reported that in 2000, more than 82% of all antibiotics were used for therapeutic and prophylaxis while only 18% was used for growth promotion in pigs (AHI, 2000).

However, with the widespread and inappropriate use of antibiotics, antimicrobial resistance among pathogenic bacteria has emerged and it has raised concerns about antibiotic use in animals and humans. Since the 1960's, the development of drug resistance of enteric bacteria in animals fed antibiotics has been repeatedly reported (Mitsuhashi et al., 1961; Watanabe, 1963; Smith, 1975). Animals and humans are linked through direct contact of food and water, resulting in the transmission of antibiotic resistance genes between bacteria. The antimicrobial-resistant bacteria in food animals can be transferred to humans through consumption of meat and milk products or contaminated raw vegetables, which occurs when soil is fertilized with animal manure (Teuber, 2001). The outbreaks of multidrug-resistant *Salmonella enterica* serotype Typhimurium in Denmark and the United States were the evidence of this (Cody et al., 1999; Mølbak et al., 1999; Centers for Disease and Prevention, 2001). Two patients died due to consumption of pork containing resistant *Salmonella* Typhimurium DT104 in Denmark. Thus, minimizing antibiotic usage, especially in food animals such as pigs, is a must in agriculture.

1.5 Proposed alternatives of antibiotics

Since 2006 in Europe and since 2011 in South Korea, the use of antibiotics in animal feeds has been prohibited and it can be expected that this policy will be expanded to other countries. The bans leave the animal industry, especially swine industry, looking for alternatives to antibiotics. Various natural materials, including probiotics, prebiotics, organic acids, bacteriocins, minerals, essential oils and zinc oxide, have been studied as alternatives to treat/prevent enteric diseases in pigs. Probiotic strains of bacteria have shown specific beneficial effects but the mechanisms responsible for the benefits are not known (reviewed by Vondruskova et al., 2010 and Seal et al., 2013). Prebiotics have been shown to have pathogen adhesion properties; however, this has not yet been studied in animals (Chen et al., 2014). Bacteriocin-producing probiotic bacteria have been studied in animals but the results were discouraging (Riboulet-Bisson et al., 2012). Because of the inconsistent results produced by these compounds, at this time there is no perfect alternative to antibiotics. The alternatives have been reviewed and their mechanisms of action and potential benefits in pigs are summarized in Table 1-2.

Table 1-2. Proposed alternatives of antibiotics used in swine industry. [a]

Alternatives	Mode of action	Benefits on pigs	Influence factors
Probiotics	Nutritient availability; Stimulation of gastrointestinal immunity; Increased gut resistance to infectious diseases by competing with pathogens for binding sites in the mucosa; Produce organic acids and antimicrobial compounds to inhibit the growth of pathogenic microorganisms.	Prevent or reduce the severity of infections; Modulate intestinal microbiota; Improve health conditions and growth performance; Enhance nutrient digestibility.	Combination of selected bacterial genera and their doses; Interactions of probiotics with pharmaceuticals, feed composition, storage and feed technology.
Prebiotics	Competitive exclusion to prevent the colonization of pathogenic bacteria; Selective increase in the growth of beneficial bacteria like *Lactobacillus* and *Bifidobacterium*; Influence production of volatile fatty acids, lactic acid and ammonia in the gut.	Support the growth and/or activities of probiotic bacteria in the gut; Improve feed efficiency; Prevent and treat enteric disorders.	N/A
Organic Acids	Decrease the pH in the stomach to inhibit the proliferation of pathogens; Penetrate the bacterial cell walls and destroy the specific bacteria.	Improve digestibility, nutrient resorption and growth performance of pigs; Decrease the number of pathogens in stomach and small intestine.	Concentration of organic acid and their pH; High amount of organic acids in feed can cause the reduction of feed intake, body weight gain and growth efficiency.
Bacteriocin/ Antimicrobial peptides	Interact with membranes of the target bacteria and disturb the function of membrane that leads to cell lysis or formation of membrane pores; Inhibit the synthesis of protein and RNA.	Exert antimicrobial activity without developing antimicrobial resistance; Have beneficial effects on pig performance, nutrient digestibility, intestinal microbiota and morphology.	N/A

Clay minerals	Bind to toxic materials such as aflatoxins, plant metabolites, heavy metals, and toxins by absorption capacity and reduced biological availability and toxicity for the animals; Immobilize anti-nutritional compounds in feeds, decrease pathogen numbers and depress the activity of bacterial enzymes.	Improve clinical manifestations of diarrheal diseases; Increase body weight gain and feed conversion.	Chemistry, exchangeable ions, structure and surface properties of the minerals; pH, dosage and exposure time.
Essential oil/Plant extracts	Change lipid solubility of membranes and inactivate pathogens. Oils containing phenolic compounds tend to have greater antimicrobial activity. Garlic extract is considered to be the most effective antimicrobial agent in plant extracts.	Antimicrobial, anti-inflammatory, anti-oxidative and anti-parasite.	Chemical composition and concentration
Zinc oxide	Stabilize the sensitive intestinal mucosa; Regulate the cellular signaling pathways; Involved in protein expression associated with glutathione metabolism and oxidative stress in the gut; Enhance intestinal barrier function and immune response against bacterial infections.	Reduce incidence of diarrhea and improve growth rates.	Dosage and the delivery methods

a) Based on reviews on research into alternatives to antibiotics (Vondruskova et al., 2010; Heo et al., 2013; Seal et al., 2013; Thacker, 2013).

1.6 Feed fermentation used in pigs

Traditional food fermentation was used for food preservation and preparation that provides food a variety of flavors, tastes, textures, and nutritional and therapeutic values (Mehta et al., 2012). Fermented feed especially the liquid feed used in swine production have gained great interest in recent years due to four reasons: 1) limit or prohibit use of antibiotics in swine production; 2) money saving by using cheap ingredients for fermentation; 3) the increasing liquid co-products from the bioethanol industry after the production of biofuels are suitable for feeding; 4) an environmental friendly way to avoid waste disposal by using the co-products from the food/pharmaceutical/biofuel industry (Canibe & Jensen, 2012). Besides, fermented feed can also benefit the pigs by reducing the risk of enteric diseases. Pigs fed with fermented liquid feed had lower number of coliforms in the gastrointestinal tract or in feces compared to pigs fed with non-fermented feed (Mikkelsen & Jensen, 2000; Moran, 2001; van Winsen et al., 2002; Hong et al., 2009). Many studies have related the used of fermented feed to the reduced prevalence of Salmonella (Bahnson et al., 2006; Farzan et al., 2006; Poljak et al., 2008). The effects of feeding fermented feed on development of porcine proliferative enteropathy, swine dysentery and porcine intestinal spirochaetosis have also been studied in recent years (Boesen et al., 2004; Lindecrona et al., 2003; Lindecrona et al., 2004). However, the effects of specific metabolic properties of fermentation microbiota on the beneficial effect of fermented feed have not been evaluated.

1.7 Hypothesis and objectives

The research for this thesis aimed to investigate the effects of exopolysaccharides (EPS) synthesized by *L. reuteri* in wheat fermentation on gastrointestinal microbiota (particularly the pathogen load) and to study the effects of *L. reuteri*, EPS and

reutericyclin (RTC) presented in fermented wheat diets on the fecal microbial composition in weanling pigs. The animal feeding trial to detect the potential of EPS synthesized by *L. reuteri* active against pathogen *E. coli* in weanling pigs will be described and discussed in chapter two. Chapter three will focus on the effects of *L. reuteri*, EPS and reutericyclin on fecal microbiota of weanling pigs. A general discussion and conclusion will be presented in chapter four.

1.8 References

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2. Exopolysaccharides synthesized by *Lactobacillus reuteri* in fermented feed have positive effects on protecting against *E. coli* in weanling pigs

2.1 Introduction

During the early weaning period of pigs, the stress of weaning and the movement to a different environment increases the potential for poor growth performance, nutrient malabsorption and even diseases (Kohler & Moon, 1984; Barnett et al., 1989; Hedemann & Jensen, 2004). Diarrhea caused by pathogenic *Escherichia coli,* especially Enterotoxigenic *E. coli* (ETEC), is an important disease of pigs, which may result in considerable financial loss in the swine industry. ETEC strains are known to possess proteinaceous appendages or specific fimbriae for intestinal attachment and colonization (Parry & Rooke, 1985), which are the first steps in establishing an enteric infection (Nagy & Fekete, 2005). After the initial colonization, ETEC produce proteins (enterotoxins) and induce severe watery diarrhea by increasing secretion and reducing absorption of enterocytes and water in intestine (Gyles, 1994; Blanco et al., 1997). For many years, antibiotics have been the first line of defense against this infectious disease (Barton, 2000); however, with increasing concerns about antimicrobial resistance and limited or prohibited use of antibiotics, alternatives such as probiotics, prebiotics, organic acids and anti-adhesive glycans have been suggested to treat/prevent *E. coli* infections (Fairbrother et al., 2005; Nagy & Fekete, 2005). In-feed probiotic LSP 122 was reported to decrease the incidence and severity of ETEC caused diarrhea (Kyriakis et al., 1999); probiotic *P. acidilactici* or *S. cerevisiae boulardii* and neoglycans of porcine albumin conjugated with prebiotic galactooligosaccharides were shown to reduce ETEC attachment to the intestinal

mucosa of pigs (Daudelin et al., 2011; Sarabia-Sainz et al., 2013); and organic acid, especially lactic acid, were also proved to be effective in controlling post-weaning diarrhea in pigs (Tsiloyiannis et al., 2001).

Although these studies have demonstrated some positive effects of feed additives on ETEC, gut health and development of pigs, the replacement of antibiotics by any single substance still seems unlikely (De Lange et al., 2010), A combination of substances may be a more effective strategy (Pluske, 2013), and feed fermentation have the potential to achieve this. During feed fermentation with Lactic Acid Bacteria (LAB), probiotic LAB strains, organic acids and prebiotic exopolysaccharides (EPS) could all be present and could produce a combined effect. However, to our knowledge, few studies have been conducted in this way. Wang et al. (2010) found that EPS synthesized by *Lactobacillus reuteri* inhibited K88 (F4) antigen-positive ETEC strains that bind to porcine erythrocytes in hemagglutination assays. Recent findings by Chen et al. (2014) confirmed the anti-adhesive properties of these EPS in an *in vivo* small intestinal segment perfusion model. Thus, a feeding trial to evaluate anti-adhesive ability and potential application of EPS to control ETEC infection is justified. Because EPS are produced by LAB during fermentation in the presence of sucrose (Gänzle & Schwab, 2005; Gänzle & Schwab, 2009), feed fermentation was a good choice for our study.

Many studies used infection models such as ETEC challenge to check the effects of feed additives on ETEC (McKenzie et al., 2008; Jansman et al., 2010; Kiarie et al., 2010; Daudelin et al., 2011). The infection model is helpful to check the specific effects of additives against specific ETEC strains but it cannot guarantee the control of all ETEC infections because the types of ETEC causing infections vary in different

area (Fairbrother et al., 2005). Therefore, to make our study closer to commercial application, a non-infection model was used.

In this study, a mixture of basal diets and fermented feed containing EPS was used. *L. reuteri* TMW 1.656 and *L. reuteri* LTH 5794 were used to ferment wheat with and without sucrose. The basal diet supplemented with organic acid, to reach the same pH as fermented feed, were used as a reference group. The aim of our study was to assess the anti-adhesive capacity of EPS produced by *L. reuteri* and the probiotic properties of *L. reuteri* in pigs. Because of the high ratio of water and feed in pigs, many studies were done only with fermented liquid feed (Canibe & Jensen, 2012). Our study will provide an alternate approach to investigating the effects of fermented feed in pigs. *L. reuteri* TMW 1.656 used in this study also produces reutericyclin, a low molecular weight antibiotic substance (Höltzel et al., 2000), that is reported to inhibit gram-positive bacteria (Gänzle, 2004). The effects of reutericyclin on weaning pigs will also be investigated.

2.2 Materials and methods

2.2.1 Feed preparation

2.2.1.1 Microorganisms and growth condition

Reuteran-forming *Lactobacillus reuteri* TMW 1.656 and levan-forming *L. reuteri* LTH 5794 in the presence of sucrose (Tieking et al., 2003; Gänzle & Schwab, 2005; Kaditzky et al., 2008; Gänzle & Schwab, 2009) were obtained from the culture collection of the Food Microbiology laboratory at the University of Alberta. Working cultures were prepared from glycerol stock stored at -80 °C. Strains were streaked on modified MRS agar (Meroth et al., 2003) and incubated anaerobically at 37°C for 48 h. Single colonies were picked from these plates and sub-cultured twice in modified MRS broth.

2.2.1.2 Fermentation optimization and feed fermentation

Wheat flour (provided by University of Alberta Swine Research and Technology Center) was mixed with an equal amount of tap water. The mixture was inoculated with cells from overnight cultures that were washed with sterile tap water and added to reach cell counts of approximately 10^7 CFU (colony forming unit) g^{-1}. After 24 h fermentation at 37°C, 90% w/w of the contents were replaced with fresh wheat flour in water and subsequently fermented under the same conditions. After 4 subsequent flour-water replacements, fermented feed was prepared again from a freshly prepared working culture.

For in-situ production of EPS, *L. reuteri* LTH 5794 and TMW 1.656 fermented with the addition of 10% (w/w) and 20% (w/w) sucrose were evaluated (Table 2-1). After comparing the production of EPS and other metabolites, *L. reuteri* fermented feed was prepared with the addition of 10% (w/w) sucrose. Wheat fermented with the same strains but with the addition of 5% (w/w) glucose and 5% (w/w) fructose in place of sucrose, served as EPS-negative controls. Additionally, a chemically-acidified control was prepared with 5% (w/w) fructose, 5% glucose (w/w), 4 parts of lactic acid (80%) and 1 part of glacial acidic acid (100%) to reach a pH of 3.8.

Table 2-1. Metabolites formed by *L. reuteri* after 24 hours of wheat feed fermentation.

***L. reuteri* strains**	**Sucrose added (%)**	**pH**	**Lactate***	**Acetate***	**Ethanol***	**EPS****
TMW 1.656	10	3.7	87.1±5.8	51.0±4.2	36.2±1.8	5.6±1.0
TMW 1.656	20	3.7	79.4±0.1	71.3±1.3	2.5	3.7±1.1
LTH 5794	10	3.7	80.7±4.6	57.3±3.7	27.31±4.9	3.2±0.6
LTH 5794	20	3.7	70.7±3.3	67.1±3.4	3.41±4.8	3.7±0.6

pH of wheat dough at the start of the fermentation: 6.1. * indicate the unit is mmol/ kg dough; ** indicate the unit is g/kg dough. Data was provided by Dr. Sandra Galle.

After 24 h of fermentation, organic acids and EPS quantification was determined as previously described (Galle et al., 2010). Data were obtained from two separate wheat fermentations. To verify that the strains used to inoculate fermented feed dominated the fermentation, pH was measured after 24 h of each fermentation. Serial dilutions of each fermented feed were done and plated on MRS agar plates to confirm the persistence of the starter cultures.

2.2.2 Animals and diets

The animal study was approved by the Animal Care and Use Committee, according to the guidelines of the Canadian Council on Animal Care, and the study was done at the University of Alberta Swine Research and Technology Centre (SRTC), Edmonton, AB, Canada. A total of 36 crossbred, castrated male pigs were selected at weaning (~21d of age). Each pig was housed in an individual pen (0.5×1.22 m) in a temperature-controlled room (28 ± 2.5 ºC) during the three-week experimental period. The pigs were divided into 6 consecutive and similar blocks with 6 pigs in each. One pig per block was assigned to one of six experimental diets (Table 2-2) for a total of 6 observations per diet. Control (CTRL) and acidified (ACID) diets contained unfermented wheat while the other four diets (REU+, REU-, LEV+ and LEV-) contained fermented wheat. The diet was formulated to meet or to exceed National Research Council Canada (NRC) (2012) nutrient recommendations for 5 to 10 kg pigs (Table 2-3). 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. The pigs were given free access to feed from a pen feeder and water from a pen cup

drinker. They were fed two equal meals in mash form twice daily (at 8 am and 4 pm). At the end of the trial, the pigs were fed their last 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 day 0, 7, 14 and 21. Feed intake was measured each day. The data were used to determine average daily weight gain (ADG), average daily feed intake (ADFI) and feed efficiency (G:F).

Table 2-2. Components of experimental diets used in this study.

Components \ Diets	CTRL	ACID	TMW1.656		LTH 5794	
			REU+	REU-	LEV+	LEV-
Acids	-	+	+	+	+	+
L . reuteri	-	-	+	+	+	+
Reutericyclin	-	-	+	+	-	-
Reuteran	-	-	+	-	-	-
Levan	-	-	-	-	+	-

'+' means component present in the diet, '–' means component not present in the diet.

Table 2-3. Composition of experimental diets, as-fed basis

Ingredients	Phase 1 diet (%) (Day 0 – Day 6)	Phase 2 diet (%) (Day 7 – Day 21)
Fermented Wheat, ground (NRC[1])	20.00	50.00
Corn (NRC)	31.54	1.76
Lactose	15.00	10.00
Soy protein conc HP300	3.00	2.50
Herring meal – WCRL	6.00	2.50
B. napus canola meal (NRC)		5.00
Wheat DD GS (Lloyd)		5.00
Soybean meal (NRC)	15.00	15.00
Canola oil (NRC)	4.00	3.40
Other vitamin and mineral ingredients	5.46	4.84
Total	**100**	**100**

[1] NRC National Research Council Canada.

2.2.3 Sample collection and preparation

Fresh feces were collected in a plastic bag by hand from each pen floor on days 0, 7, 14 and 21. Feces were stored in a freezer at -20°C. Samples of gut digesta were collected from the stomach, terminal ileum, cecum and mid-colon in sterile plastic containers after the pigs were euthanized. Samples were stored at -20°C.

Frozen fecal and gut digesta samples were thawed and mixed aseptically by 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 were aseptically excised (about 5 cm). The segments were opened longitudinally and the mucosa was removed by scraping with a flame-sterilized metal spatula (Said et al., 1989). The mucosal scrapings were stored in individual tubes at -80°C for bacterial analysis.

2.2.4 Genomic DNA extraction for qPCR

Total bacterial DNA was extracted from fecal and gut digesta samples using QIAamp® DNA stool Mini kit (50) (Qiagen, Inc., Valencia, CA, USA), 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 at room temperature (approximately 20°C) at 20,000 x g for 1 min, the supernatant was incubated with an InhibitEx tablet provided in the kit to absorb DNA-damaging substances 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 DNA, and the mixture was applied to QIAamp spin columns provided in the kit. DNA was dissolved in buffer AE (Qiagen) after washing with buffers AW1 and AW2 (Qiagen).

DNA concentrations were measured by Nano-Drop spectrophotometer system ND-1000 (Thermo Fisher Scientific Inc., Wilmington, USA), and purity was assessed by determining the ratio of absorbance at 260 and 280 nm. DNA samples were required to have 260:280 nm ratios > 1.8 for further testing. Sample preparations with a ratio less than 1.8 were repeated.

2.2.5 PCR primers

All of the primers used in this study and their target organisms are listed in Table 2-4. Primers GTFA F and GTFA R are specific for detection of reuteransucrase (*L. reuteri* TMW 1.656). Primers used for levansucrase (*L. reuteri* LTH 5794) detection were designed as follows: Primers FTF F and R were designed in Primer 3 (Rozen, 1999) using sequence of levansucrase in strain *L. reuteri* SD2112 (Accession number: NC_015697). PCR amplicons of the two experimental strains (*L. reuteri* LTH 5794 and TMW 1.656) obtained with FTF F and FTF R primers were purified from agarose gels and sequenced by Sanger sequencing (Macrogen, Rockville, MD). The sequence data were aligned with CLUSTAL-W (Thompson et al., 1994) to find a unique sequence of *L. reuteri* LTH 5794 and to design primers LEV F and LEV R in Primer 3. Basic Local Alignment Search Tool (BLAST) was used to check the theoretical specificities of the primer sequences (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Primers were synthesized by Integrated DNA Technologies Inc. (http://www.idtdna.com/site), diluted to a final concentration of 10 µg of primer per µL with autoclaved MilliQ water upon receipt and stored at -20°C.

Table 2-4. Primers used to profile the microorganism in fecal and gut digesta samples.

Target organism	Primer	Sequence (5'-3')	Product size (bp)	Tm** (°C)	Reference
Total bacteria	HDA F	ACTCCTACGGGAGGCAGCAGT	199	64	(51, 52)
	HDA R	GTATTACCGCGGCTGCTGGCAC			
Lactobacillus group*	Lacto F	TGGAAACAGRTGCTAATACCG	231-233	64	(53)
	Lacto R	GTCCATTGTGGAAGATTCCC			
Lactobacillus reuteri	Lreu F	CAGACAATCTTTGATTGTTTAG	303	64	(54)
	Lreu R	GCTTGTTGGTTTGGGCTCTTC			
Reuteransucrase	GTFA F	AATTAAACTGGTTATACTATCTC	160	57	(18)
	GTFA R	GAGTTCATACCATCTGCAGC			
Levansucrase	FTF F	TATCAATGATACAAATAATGC	1065	56	In this study
	FTF R	GCGTTTCAGGATCATTTGGT			
	LEV F	GTCAATTTGATCCTTCGCC	460	57	In this study
	LEV R	TGCAACTAAGGAAATTAAGGGC			
Reutericyclin	RC F	GGCGGAACGTTGAATATTGT	248	60	In this study
	RC R	ATTTTGGGGGAATCATAGCC			
Universal stress protein A	Ecoli F	CCGATACGCTGCCAATCAGT	884	66	(55)
	Ecoli R	ACGCAGACCGTAGGCCAGAT			
Heat labile toxin	LT F	CCGTGCTGACTCTAGACCCCCA	480	68	(56)
	LT R	CCTGCTAATCTGTAACCATCCTCTGC			
Heat stable toxin a	STa F	ATGAAAAAGCTAATGTTGGC	193	65	(57)
	Sta R	TACAACAAAGTTCACAGCAG			
Heat stable toxin b	STb F	TGCCTATGCATCTACACAAT	113	60	(58, 59)
	STb R	CTCCAGCAGTACCATCTCTA			
Fimbriae K88	K88 F	GCACATGCCTGGATGACTGGTG	439	67	(60)
	K88 R	CGTCCGCAGAAGTAACCCCACCT			

**Lactobacillus* group includes *Lactobacillus*, *Pediococcus* and *Weissella* spp.

** indicate the annealing temperature used in this study for PCR.

2.2.6 Quantitative PCR

Quantitative PCR (qPCR) was done on a 7500 Fast Real-Time PCR System (Applied Biosystems model, Foster City, CA, USA). The total reaction volume of 20 μL contained 10 μL of Quanti Fast SYBR green master mix (Applied Biosystems), 2 μL (10 μM) of primer, 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 approximately 100 mg/L.

Standard curves were generated using serial ten-fold dilutions of the purified PCR amplicons, which was amplified from specific primers (Table 2-4) and genomic DNA from *L. reuteri* strains or pig feces. The initial concentration of purified PCR amplicons was determined by Nano-Drop spectrophotometer system ND-1000. Amplification conditions generally involved 1 cycle at 95°C for 5 min for initial denaturation, 40 cycles of denaturation at 95°C for 30s, annealing with optimal annealing temperatures (Table 2-4) for 30s, and extension at 72°C for 30s. In the melting curve stage, the reaction conditions included 1 cycle of 95°C for 15s, 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 ensure correct amplification results, the melting curves were checked to determine if there was a single peak in the results after amplification.

2.2.7 Statistical analysis

Data were analyzed using Statistical Analysis System (SAS version 9.3; SAS Institute, 2012). Data for fecal bacteria and growth performance were analyzed according to randomized complete block design with repeated measurement using the Mixed Procedure (Proc MIXED). The model included diet, period and interaction of diet and period as fixed effects. Block and pigs were considered as random effects. Data for microbiota of gut digesta were subjected to ANOVA as a randomized completely block design using the GLM procedure (Proc GLM). Treatment comparisons were determined by contrast. The experimental unit was pig and $p<0.05$ with Bonferroni-adjusted (SAS version 9.3) was considered to be a statistically significant difference. Kolmogorov-Smirnoff test (Young, 1977) was used to test for normality of all variables. Results of growth performance are presented as means, while data for bacteria analyses are presented as least square means with standard errors.

2.3 Results

2.3.1 EPS in feed

EPS formation by the test strains was evaluated in both wheat- and corn-based. After comparison, corn flour, which is the primary energy source in pig diets, is not a good choice for fermented feed in this study. The in-situ production of EPS in corn fermentation was low compared with wheat fermentation (data not shown) no matter how much sucrose was present. No EPS was quantified/produced in the corn fermentation with *L. reuteri* LTH 5794 (data not shown). For this reason, wheat flour was used in this study for feed fermentation.

The metabolites formed after fermentation by the two strains of *L. reuteri* are shown in Table 2-1. The addition of 10% sucrose gave a higher yield of EPS compared with 20% sucrose when fermented by *L. reuteri* TMW 1.656. The EPS yield with *L. reuteri* LTH 5794 with 10 and 20% added sucrose were comparable, but the addition of 10% sucrose gave lower acetate and higher ethanol yields, which would reduce the impact of acetate on palatability of feed. Hence, wheat fermented by *L. reuteri* strains with addition of 10% sucrose was used to produce the fermented feed.

The six diets (Table 2-2) used in our study provided variable comparisons. Fermented diets containing one of the two strains can be compared between each other and find the better strain. Fermentation with or without addition sucrose help to learn the effects of EPS. Organic acid used in this study will not only ensure the comparable pH between unfermented diet and fermented diet to investigate the probiotic effects of *L. reuteri* strains, but also able to check the effects of itself in pigs. The control group provides a baseline of the experiment.

2.3.2 Growth performance of pigs

Growth performance data for the 21-day feeding trial are shown in Table 2-5. Pigs fed diets containing exopolysaccharides (REU+ and LEV+) had a lower average daily feed intake and average daily weight gain compared with pigs fed unfermented diets (CTRL and ACID). Average daily feed intake was less in pigs fed the four fermented feed containing diets than pigs fed the two unfermented diets. No significant difference was found in feed efficiency (ratio of average daily weight gain to average daily feed intake) within all treatment groups. In the entire experimental period, all pigs remained in good condition and did not develop diarrhea or show any clinical signs of disease.

Table 2-5. Growth performance of pigs fed supplemental fermented diet for 21 days.

	Dietary treatments [1]						SEM	P value				
	CTRL	ACID	TMW 1.656		LTH 5794			Acids	EPS	*L. reuteri* + EPS	*L. reuteri*	Fermentation
			REU+	REU-	LEV+	LEV-						
Diets	1	2	3	4	5	6		1 vs 2	3+5 vs 4+6	1+2 vs 3+5	1+2 vs 4+6	1+2 vs 3+4+5+6
ADFI (g/d)												
0-21	305	295	268	283	266	265	19	0.659	0.644	**0.029**	0.085	**0.025**
ADG (g/d)												
0-21	255	264	241	240	210	240	19	0.712	0.403	**0.046**	0.24	0.067
G:F												
0-21	0.76	0.84	0.86	0.79	0.74	0.84	0.05	0.231	0.628	0.894	0.725	0.899

[1] Diets were supplemented with organic acid or 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. Data provided by Hong Anh Le Minh.

2.3.3 Detection of lactobacilli and two strains of *L. reuteri* in fecal samples

To quantify the two experimental strains used in this study, qPCR with strain-specific primers are used for detection. *L. reuteri* and lactobacilli are also quantified to evaluate the effects of treatments on them. In this study, reuteransucrase and

levansucrase were detected in the fecal samples of pigs fed diets containing *L. reuteri* TMW 1.656 and *L. reuteri* LTH 5794 fermented feed, respectively [Fig. 2-1 (C and D)]. Before treatment (day 0), reuteransucrase was not detect in any samples and levansucrase was only detected in samples from pigs assigned to the control group. After the experimental diets were started, reuteransucrase was detected mainly in pigs fed *L. reuteri* TMW 1.656 fermented feed containing diets (REU+ and REU-) representing more than 6 $\log_{10}$ DNA gene copies/g feces (wet weight). Fecal samples from the pigs fed ACID diet also contained reuteransucrase (5.4 to 5.7 $\log_{10}$ DNA gene copies/g feces (wet weight)) (Fig. 2-1 [C]); Gene copy numbers of levansucrase were significantly higher in fecal samples from pigs fed the diet containing *L. reuteri* LTH 5794 fermented product (LEV+ and LEV-) than in other groups (Fig. 2-1 [D]). Levansucrase was also quantified/detected in other samples from day 14 (Fig. 2-1 [D]). Lactobacilli counts remained stable throughout the study period (ranging from 8.7 to 9.7 $\log_{10}$ DNA gene copies/g feces [wet weight]) [Fig. 2-1 (A)]. Gene copy number of *L. reuteri* increased significantly after treatment (day 7) in all groups but decreased, with exception of ACID group, at the same level as before treatment (day 0) at day 21 [Fig. 2-1 (B)]. *L. reuteri* counts remained at the same high level from day 7 to day 21 in ACID group [Fig. 2-1 (B)].

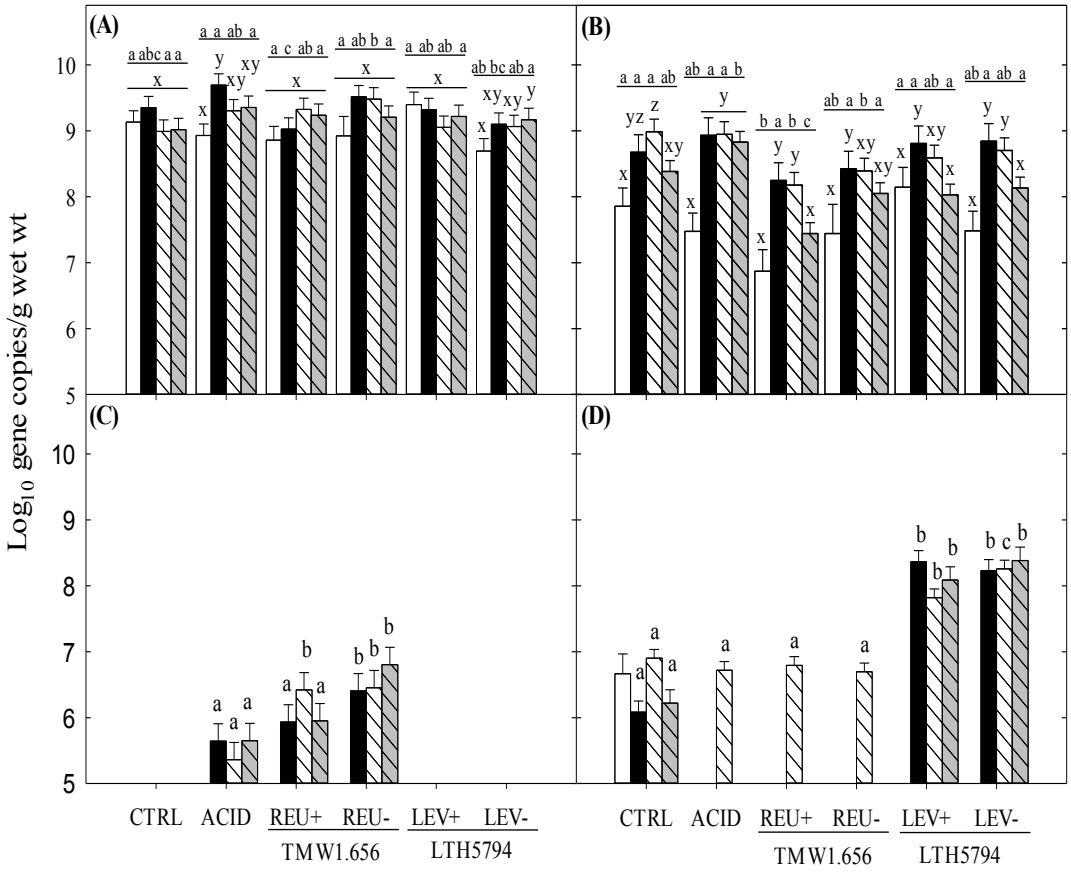

Figure 2-1. Gene copy number of (A) Lactobacilli, (B) *L. reuteri*, (C) Reuteransucrase and (D) Levansucrase in feces of pigs collected on day 0 (white bars), day 7 (dark bars), day 14 (white slash bars) or day 21 (dark slash bars). Data presented are least square means (n=36), with their standard errors represented by vertical bars. Superscripts a, b and c denote significant difference ($p<0.05$) between diets at each time point; Superscripts x, y and z denote significant difference ($p<0.05$) within diet over time. Values not having the same letter are significantly different. The detection limit for Lactobacilli, *L. reuteri* and Levansucrase was 6 $\log_{10}$ gene copies/g of feces (wet weight).

2.3.4 Detection of lactobacilli and two strains of *L. reuteri* in gut digesta

The detection of the two experimental strains in the gut digesta was the same as in fecal samples. Gene copy number of reuteransucrase (6.7 to 7.3 $\log_{10}$ DNA gene

copies/g digesta (wet weight)) was significantly higher in both REU+ and REU- group compared with other groups (Fig. 2-2 [C]). Levansucrase was only detected in pigs from LEV+ and LEV- groups, ranging from 6.8 to 8.1 $\log_{10}$ DNA gene copies/g digesta (wet weight) (Fig. 2-2 [D]). Lactobacilli counts were the same between treatments in stomach digesta (8.9 to 9.4 $\log_{10}$ DNA gene copies/g digesta [wet weight]) and in cecum and colon digesta (9.5 to 9.9 $\log_{10}$ DNA gene copies/g digesta [wet weight]) (Fig. 2-2 [A]). In digesta samples from ileum, overall lactobacilli counts (7.3 to 8.5 $\log_{10}$ DNA gene copies/g digesta [wet weight]) were at least a half-log lower than other part of the gut digesta samples. The ACID group had the lowest number (Fig. 2-2 [A]). *L. reuteri* counts were comparable between treatments in different part of the gut digesta samples with exception of the ACID group, which had significantly lower counts in stomach, ileum and cecum digesta samples than other groups (Fig. 2-2 [B]).

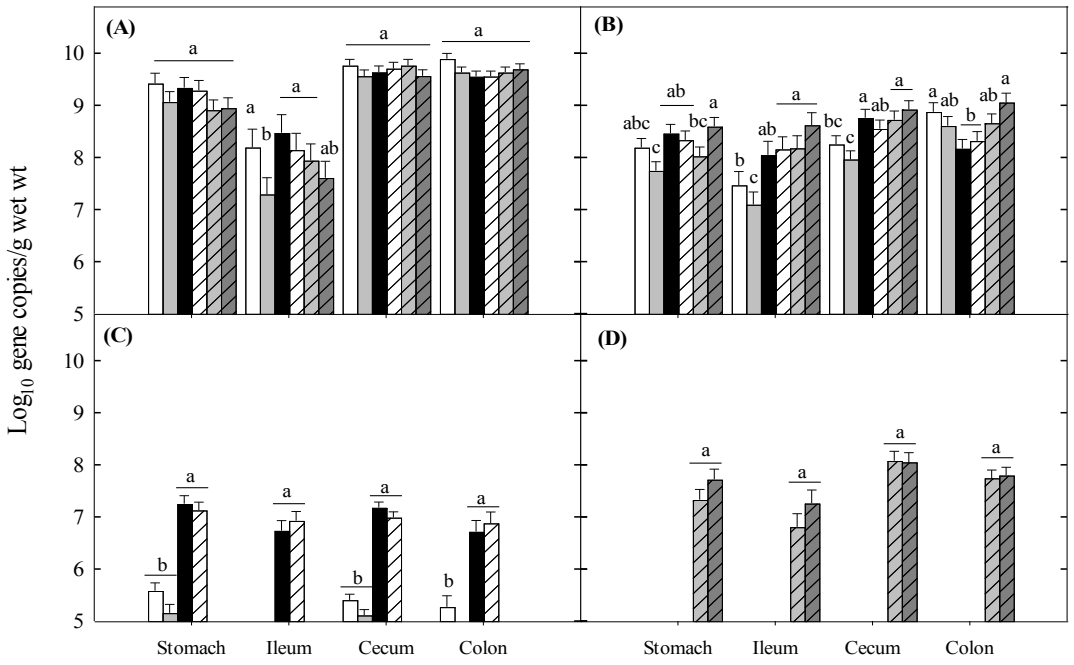

Figure 2-2. Gene copy numbers of (A) Lactobacilli, (B) *L. reuteri*, (C) Reuteransucrase and (D) Levansucrase in gut digesta of pigs fed with CTRL (white bars), ACID (light gray bars), REU+ (dark bars), REU- (white slash bars), LEV+ (light gray slash bars), or LEV- (dark gray slash bars). Data presented are as least square means (n=36), with their standard errors represented by vertical bars. Superscripts a, b and c denote significant difference ($p<0.05$) between diets at each site. Values not having the same letter are significantly different. The detection limit for Lactobacilli, *L. reuteri* and Levansucrase were 6 $\log_{10}$ gene copies/g of digesta (wet weight).

2.3.5 Detection of *E. coli* and its virulence factors

Virulence factors of ETEC as well as the total *E. coli* were quantified by qPCR. In fecal samples, *E. coli* was detected in feces of all pigs throughout the study period. At day 21, the gene copy numbers of *E. coli* were the lowest within all treatments and

REU+ group had the lowest *E. coli* count compared with other groups (Fig. 2-3 [A]). Virulence factors (STb, LT and K88) of ETEC were detected after treatment day 7; they reached a peak on day 14 and then decreased by day 21 with exception of REU+ group that decreased from day 7 to day 21. When compared between treatments on day 21, REU+ group had the lowest log count of STb (Fig. 2-3 [B]) and was the only group in which LT was not detected (Fig. 2-3 [C]). STa was below detection limit in all fecal samples (data not shown).

In gut digesta samples (ileum, cecum and colon), *E. coli* and STb were detected in pigs fed unfermented diets (CTRL and ACID) but they were not detected in pigs fed diets containing *L. reuteri TMW 1.656* fermented feed (REU- and REU+) (Fig. 2-4). Other virulence factors including LT, STa and K88 fimbriae, were not detected in any samples from gut digesta. Neither *E. coli* nor its virulence factors were detected in samples of mucosal scrapings from the jejunum.

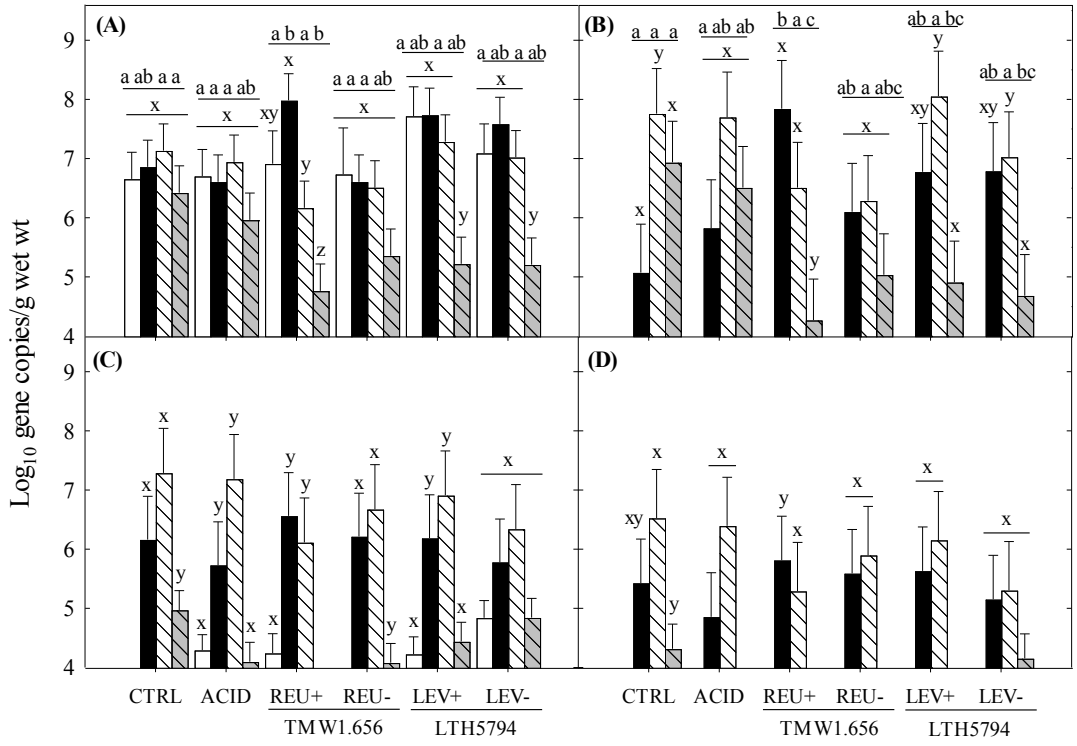

Figure 2-3. Gene copy numbers of (A) *E. coli* and its virulence factors (B) Heat stable

enterotoxin b (STb), (C) Heat labile enterotoxin (LT), (D) K88 fimbriae in feces of pigs collected on day 0 (white bars), day 7 (dark bars), day 14 (white slash bars) or day 21 (dark slash bars). Data presented are least square means (n=36), with their standard errors represented by vertical bars. Superscripts a, b and c denote significant difference ($p<0.05$) between diets at each time point; Superscripts x, y and z denote significant difference ($p<0.05$) within diet over time. Values not having the same letter are significantly different. The detection limit was 4 $\log_{10}$ gene copies/g of feces (wet weight).

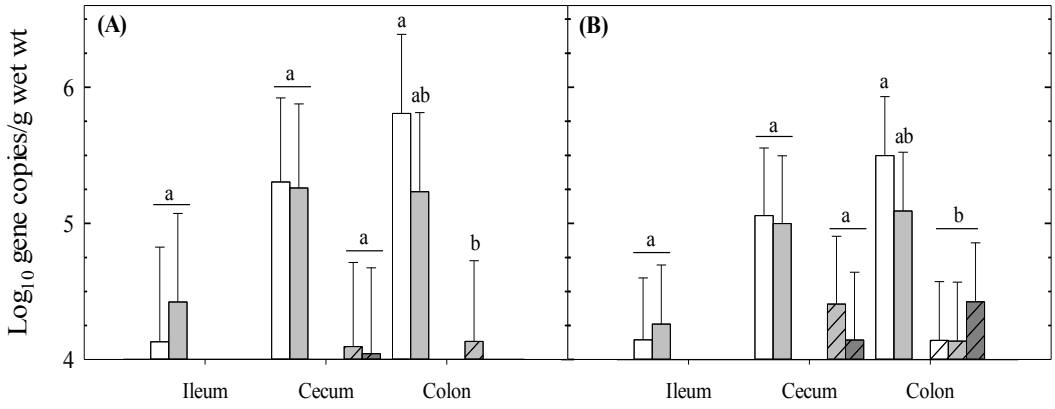

Figure 2-4. Gene copy numbers of (A) *E. coli* and its virulence factor (B) Heat stable enterotoxin b (STb) in gut digesta of pigs fed with CTRL (white bars), ACID (light gray bars), REU+ (dark bars), REU- (white slash bars), LEV+ (light gray slash bars), or LEV- (dark gray slash bars). Data presented are as least square means (n=36), with their standard errors represented by vertical bars. Superscripts a, b and c denote significant difference ($p<0.05$) 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).

2.4 Discussion

ETEC is an important cause of diarrhea in newborn and post-weaning pigs, resulting in serious morbidity and mortality (Gyles, 1994) and causing major financial loss in the swine industry (Melkebeek et al., 2013). Reuteran produced by *L. reuteri* TMW 1.656 and levan produced by *L. reuteri* LTH 5794 were previously studied for their ability to bind ETEC *in vitro* (Wang et al., 2010) and in the *in vivo* small intestinal segment perfusion model (Chen et al., 2014). Before commercial application, an animal trial was required to confirm the effects. Fermented pig feeds have received growing attention in recent years because of the potential to enhance gut health and growth performance (van Winsen et al., 2001; Canibe & Jensen, 2012). It has been reported that average daily weight gain and average daily feed intake of pigs fed fermented liquid diets was less than pigs fed unfermented feeds but feed efficiency was similar (del Rocio Amezcua et al., 2007; Canibe & Jensen, 2012). The same finding was apparent in our study; however, significantly higher average feed intake and average daily weight gain of pigs in CTRL and ACID group was observed than pigs in REU+ and LEV+ groups. This result indicated that the pigs might prefer the balanced sweet and sour flavor, because the ACID diet contained fructose, glucose and organic acids while REU+ and LEV+ groups mainly contained the organic acid after feed fermentation. This is a very interesting finding in our study.

Organic acid or acidified diets have been reported to have positive effects in weaning pigs due to its antimicrobial properties, growth promotion by lowering gastric pH and inhibition of growth of coliform bacteria (Tsiloyiannis et al., 2001; Heo et al., 2013) but such effects were not observed in our study. In our study, the levels of *E. coli* and ETEC virulence factors were the same in the CTRL and ACID groups. However, changes in the numbers of lactobacilli and *L. reuteri* in the ACID group was detected.

In fecal samples of this study, both lactobacilli and *L. reuteri* counts in ACID diet increased significantly after one week and the *L. reuteri* counts remained at a high level until the end of the experiment. These results could be explained by the carbohydrate content of the ACID diet. Glucose, fructose and organic acid (glacial acidic acid and lactic acid) mixed with unfermented wheat was fed to the pigs. When the substrates entered the stomach, a fermentation could be initiated by the autochthonous lactobacilli, which resulted in the prolification of lactobacilli. In samples from the gut digesta, the gene count of *L. reuteri* of samples of pigs fed the ACID diet was lower than in samples from animals receiving the fermented feed. This could be attributed to the high number of *L. reuteri* present in the fermented diets. A lower level of *L. reuteri* was observed in feces and colon digesta from pigs fed the REU+ and REU- diets and this might be due to the production of reutericyclin.

Reutericyclin is an antibiotic produced by some *L. reuteri* strains including *L. reuteri* TMW 1.656 used in this study. It is reported to be active against gram-positive bacteria such as *Streptococcus sp.* and *Clostridium difficile* (Hurdle et al., 2009; Hurdle et al., 2011; Cherian et al., 2014). In the present study, reutericyclin was detected in two groups of pigs that were fed the diet with *L. reuteri* TMW 1.656 and the gene copy number was around 10^7 in log (data not shown). However, effects of reutericyclin on *Clostridium difficile* were tested but the results were not noted in the study (data not shown).

Real-time quantitative PCR (qPCR) is a rapid and reliable method to detect bacteria in fecal and gut digesta samples (Martin, 2012). Strain-specific primers (GTFA and LEV in Table 2-3) used in this study had their limitations, because they could only detect a small component/fraction of the bacterial species or metabolites believed to be present in gut and feces of pigs (Table 2-3). Small amounts of reuteransucrase and

levansucrase were detected in pigs that were not fed the test strain (reuteransucrase in ACID and levansucrase in CTRL); however, the methods are still adequate for differentiation between the test and wild-type strains. According to the qPCR data, the two *L. reuteri* test strains persisted in the gut, were, excreted in the feces and accounted for a high percentage of the total lactobacilli in both gut digesta and fecal samples.

Feeding *L. reuteri* strains had no apparent influence on the autochthonous lactobacilli but inhibited growth of *L. reuteri* and *E. coli*. Previous studies showed that levels of fecal lactobacilli were not influenced when pigs were fed lactobacilli (Du Toit et al., 1998; De Angelis et al., 2007). Our study confirmed this. However, we observed a decrease of *L. reuteri* counts in samples from pigs fed fermented diets, which differed markedly from the other studies that indicated a significant increase of *L. reuteri* in feces after intake of lactobacilli (Wolf et al., 1995; Shornikova et al., 1997; Valeur et al., 2004). Decreased detection of fecal *Enterobacteriaceae* and coliform bacteria after administration of LAB was reported (Shornikova et al., 1997). In the present study, we checked the effect of *L. reuteri* on *E. coli* and ETEC and found that the growth of *E. coli* and ETEC was inhibited in pigs fed *L. reuteri* fermented diets, which indicated a health benefit for the two experimental strains. On the basis of the definition of probiotics: “live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host” (WHO 2001), the results in this study could be evidence for the promotion of *L. reuteri* strains from gut symbiont to probiotic (Frese et al., 2013; Chen et al., 2014).

EPS have been suggested as anti-adhesive compounds involved in reduction of bacteriophage attack (Forde & Fitzgerald, 1999) and adhesion to mucosal surfaces (Rozen et al., 2004), as well as adhering to pathogenic bacteria such as *E. coli* (Ruas-

Madiedo et al., 2006). In this study, EPS-positive diets revealed lower gene copy numbers of *E. coli* and ETEC virulence factor in samples when compared with EPS-negative diets (REU- and LEV-), which might be attributable to the anti-adhesive properties of EPS. REU+ diet also resulted in the lowest gene count and showed a decreasing trend of *E. coli* and ETEC virulence factor. These results further supported the *in vitro* (Wang et al., 2010) and *in vivo* findings (Chen et al., 2014) that reuteran was more effective than levan in preventing ETEC adhesion. However, the comparisons between REU+ and REU- or LEV+ and LEV- are insignificant. Possible explanation would be: a) the dose of EPS present in the feed and the amount that the pigs ingested; b) EPS reached in the gut might not only react with ETEC strains, they might also have effects on other gut bacteria; c) wheat starch, and the addition glucose and fructose present in REU- and LEV- diets would probably be fermented in the gut of swine and give the potential production of EPS.

This feeding trial further confirmed the anti-adhesive properties of reuteran that were observed *in vitro* and *in vivo*. All studies, including *in vitro*, *in vivo* and this feeding study, demonstrated that reuteran has the potential application to prevent or control ETEC infections in swine. Without a specific bacterial challenge study, our animal trial and the conditions that we used are much closer to conditions that might occur in the swine industry and the results can be easily translated into real application. Thus, I conclude that diets supplemented with *L. reuteri* TMW 1.656 fermented feed that produce reuteran is promising for use in the swine industry.

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3. Effects of reutericyclin on fecal microbiota in weanling pigs

3.1 Introduction

Gut microbiota of pigs undergo a major transition after weaning (Konstantinov et al., 2004; Lallès et al., 2007). The microbiome of suckling pigs is dominated by lactobacilli (Konstantinov et al., 2006a) while strict anaerobic fibre-fermenting organisms in the phyla *Firmicutes* and *Bacteroidetes* dominate the microbiome of growing pigs (Lamendella et al., 2011; Riboulet-Bisson et al., 2012). With the stress from the change of environment and diet, the transition after weaning is characterized by a decreased abundance of protective lactobacilli (Konstantinov et al., 2006a), and this provides opportunity for overgrowth of pathogenic bacteria, including enterotoxigenic *Escherichia coli* (ETEC). Post-weaning diarrhea (PWD) is the typical disease caused by ETEC infection and results in major financial losses in swine industry.

Feed additives affect the gut microbiota of pigs. Probiotics were reported to influence gut microbiota communities by decreasing pathogenic bacteria and improving the gut health (Le Bon et al., 2010; Zhang et al., 2010; Bednorz et al., 2013). *Lactobacillus sobrius/amylovorus*, a porcine intestinal commensal (Konstantinov et al., 2006b), reduced the level of *E. coli* F4 in the gut (Konstantinov et al., 2008) and did not change the microbial community of the hindgut (Su et al., 2008). The mechanisms of probiotic action are likely to be multifactorial (Tuohy et al., 2003). Their metabolites such as exopolysaccharides (prebiotics), organic acids and bacteriocins have been found to be associated with the health-promoting properties. Exopolysaccharides (EPS) synthesized by *L. reuteri* prevented hemagglutination by ETEC (Wang et al., 2010) and protected against ETEC infection (Chen et al., 2014). Organic acids

modulate intestinal microbiota (Piva et al., 2002) and decrease the proliferation of coliform bacteria (Namkung et al., 2004). The influence of antibiotics and bacteriocins produced by probiotics on gastrointestinal bacteria of pigs were also reported in recent years (Jensen, 1998; Shim et al., 2005; Pluske, 2013; Looft et al., 2014; Rea et al., 2014).

However, most of these studies were targeted to elucidate mechanisms of feed additives on specific bacterial strains or groups but few of them have revealed their effects on the diversity of the microbiome. A recent study of the influence of a probiotic and its bacteriocin on fecal microbiota community (Riboulet-Bisson et al., 2012) indicated a significant difference between the bacteriocin-positive and the control group but no significant difference between bacteriocin-negative group and the two groups. The authors attributed the significant results to bacteriocin production; however, this conclusion cannot be generalized and further study is required.

Reutericyclin is a naturally-occurring tetramic acid produced by strains of *L. reuteri* (Höltzel et al., 2000). It is a small non-peptide substance that is active against Gram-positive bacteria by dissipating the bacterial transmembrane (Gänzle et al., 2000; Yendapally et al., 2008). *Clostridium difficile* and *Clostridium perfringens* were inhibited by reutericyclin (Hurdle et al., 2011; Cherian et al., 2014) but this activity has not been studied in animal trial. *L. reuteri* TMW 1.656, which was used in this study, is closely related to strains of *L. reuteri* TMW1.106 and LTH2584 and it produced reutericyclin during fermentation (Gänzle & Vogel, 2003; Su et al., 2012). Therefore, the effects of reutericyclin on gut microbiota especially on *C. difficile* and *C. perfringens* was evaluated.

Traditional culture-dependent microbiological methods have been used to characterize the intestinal microbiota of pigs (Salanitro et al., 1977; Allison et al., 1979; Robinson

et al., 1981; Robinson et al., 1984). With the improvement of gene sequencing technology and bioinformatics tools, 16S gene tag sequencing has been used to unveil the intestinal bacterial community. The data revealed a complex microbial community that cannot be comprehended by classic culturing methods in pigs (Simpson et al., 2000; Leser et al., 2002). The composition of the intestinal microbiota of weanling pigs is still poorly understood. Thus, the aim of our study was to reveal effects of probiotics in fermented feed with the production of exopolysaccharides and reutericyclin on the fecal bacterial composition of weanling pigs and to provide information for managing the PWD in the swine industry. Illumina-sequencing was used to obtain sequence information and Quantitative Insights Into Microbial Ecology (QIIME) pipeline was used to analyze the sequences.

3.2 Materials and methods

3.2.1 Feed fermentation and diets preparation

Complete dietary information was described in Chapter 2. Briefly, wheat flour (provided by University of Alberta Swine Research and Technology Centre) was mixed with an equal amount of tap water and inoculated with approximately 10^7 CFU g^{-1} for fermentation. Wheat fermented by *L. reuteri* LTH 5794 and *L. reuteri* TMW 1.656 with addition of 10% (w/w) sucrose or addition of 5% (w/w) glucose and 5% (w/w) fructose were aimed to obtain reuteran-positive (REU+) or -negative (REU-) and levan-positive (LEV+) or -negative (LEV-) fermented feed, respectively. A chemically acidified control (ACID) was prepared with 5% (w/w) fructose, 5% glucose (w/w), 4 parts of lactic acid (80%) and 1 part of glacial acidic acid (100%) to reach a pH of 3.8. Basal diets were mixed with 20% to 50% fermented or acidified wheat to produce the experimental feeds. Control diet (CTRL) was obtained by a mixture of basal diet and unfermented wheat. All diets were formulated to meet or

exceed nutrient recommendations of National Research Council Canada (NRC) (2012) for 5 to 10 kg pigs. Titanium dioxide (TiO_2) was added to each of the test diets as an indigestible marker.

Table 3-1. Experimental diets used in this study.

Components \ Diets	CTRL	ACID	TMW1.656		LTH 5794	
			REU+	REU-	LEV+	LEV-
Acids	-	+	+	+	+	+
L . reuteri	-	-	+	+	+	+
Reutericyclin	-	-	+	+	-	-
Reuteran	-	-	+	-	-	-
Levan	-	-	-	-	+	-

+ means component present in the diet, - means component not present in the diet.

3.2.2 Animals and experimental design

This animal trial was approved by the University of Alberta Animal Care and Use Committee under the guidelines of the Canadian Council on Animal Care and was conducted at the University of Alberta Swine Research and Technology Centre, Edmonton, AB, Canada. A total of 36 crossbred castrated weaning male pigs (~21d of age) were selected and housed in a temperature-controlled room (28 ± 2.5 ºC). Pigs were divided into 6 consecutive and similar blocks with 6 pigs per block and one pig per pen (0.5×1.22 m). One pig per block was assigned to one of the six diets (Table 3-1) for a total of 6 observations per diet. Pigs were offered *ad libitum* food and water intake allowing for adequate growth. Meals in mash form were provided in equal amount twice daily (at 8 am and 4 pm). Fresh fecal samples were collected from each pen floor in a plastic bag by hand at 0 (Week 0), 1 (Week 1), 2 (Week 2) and 3 (Week 3) weeks after weaning. A total of 137 samples were collected and stored at -20°C. Frozen fecal samples were thawed, mixed aseptically by spatula and 2 to 3 gram subsamples were stored at -80°C for bacterial analysis.

3.2.3 DNA extraction

Bacterial DNA was extracted from fecal samples using QIAamp® DNA stool Mini kit (50) (Qiagen, Inc., Valencia, CA, USA), following the manufacturer's instructions. Fecal DNA was quantified by Nano-Drop spectrophotometer system ND-1000 (Thermo Fisher Scientific Inc., Wilmington, USA). DNA quality was assessed by determining the ratio of absorbance at 260 and 280 nm. DNA samples that had 260:280 nm ratios higher than 1.8 were used. Samples with a ratio less than 1.8 were repeated until they reached the selection criterion.

3.2.4 PCR primers and probes

All the primers and probes used in this study are listed in Table 3-2 with their target gene/organisms. Gene sequences for each *clostridia* toxin (α-, β-, β2-, entero- and ι-toxin) from different strains were collected from GeneBank (http://www.ncbi.nlm.nih.gov/genbank). The sequence data were aligned with CLUSTAL-W (Thompson et al., 1994) to find a common sequence for each toxin. Primers and probes for each toxin were then designed from the common sequence using primer 3. The theoretical specificities of the primer sequences were checked by Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Primers and probes were synthesized by Integrated DNA Technologies Inc. (http://www.idtdna.com/site). Upon receipt, the primers and probes were diluted to a concentration of 10 μg of primer per μL with autoclaved MilliQ water and stored at -20°C.

3.2.5 Quantitative PCR to quantify Clostridial clusters and toxins

Quantitative PCR (qPCR) was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems model, Foster City, CA, USA). Because of the lack of DNA

templates of toxins for PCR, gBlocks® Gene Fragments were designed from primers and probes of all toxins and synthesized by Integrated DNA Technologies Inc. Standard curves for quantification of toxin genes were generated with ten-fold serial dilutions of purified PCR amplicons, which were amplified from gBlocks® Gene Fragments with the same primer pair and probe. For quantification of eubacteria and Clostridial clusters, standard curves were generated with amplicons that were amplified from fecal DNA, also with ten-fold serial dilutions. The concentration of amplicons was determined by Nano-Drop spectrophotometer system ND-1000.

Fecal DNA was diluted to a concentration of 100 mg/L and analyzed in duplicate in a MicroAmp Fast Optical 96-well reaction plate sealed with MicroAmp Optical Adhesive Film (Applied Biosystems). For bacterial and ε-toxin detection, the master mix was Quanti Fast SYBR Green (Applied Biosystems). Taqman Fast (Applied Biosystems) was used for detection of other toxins with probes. The volume of qPCR reaction was 25μL, which contained 12.5 μL of Quanti Fast SYBR Green or Taqman Fast master mix, 4 μL (10 μM) of primer, and 2 μL of template DNA from fecal samples and 6.5μL of sterile Milli-Q water. The amplification conditions included: 1 cycle of initial denaturation at 95°C for 5 min, denaturation at 95°C for 15s for 40 cycles, primer annealing at the optimal temperatures (Table 3-2) for 30s or 45s depending on the product size, and extension at 72°C for 30s. Melting curves, which were obtained after 40 cycles, combined with agarose gel separation of amplicons were used to verify the amplification results.

Table 3-2. Oligonucleotide sequences of the primers and probes used in this study. (* Y=T/C. F- Forward, R- Reverse, P- Probe.)

Target group/gene	Primer/probe	Oligonucleotide sequence (5'-3')*	Annealing T (ºC)	Product size (bp)	Reference
Clostridium cluster Ⅰ	CI F	GTGAAATGCGTAGAGATTAGGAA	58	665	(Le Bourhis et al., 2005)
	CI R	GATYYGCGATTACTAGYAACTC			
Clostridium cluster XI	CXI F	ACGGTACTTGAGGAGGA	58	139	This study (Schwab et al., 2011)
	CXI R	GAGCCGTAGCCTTTCACT			
Total bacteria	HDA F	ACTCCTACGGGAGGCAGCAGT	62	198	(Walter et al., 2000)
	HDA R	GTATTACCGCGGCTGCTGGCAC			
C. perfringens α toxin (*cpa*)	CPα F	CTTGGAGAGGCTATGCACTATTT	60	90	This study
	CPα P	6FAM-CCATATCATCCTGCTAATGTTACTGCCGT-TAMRA			
	CPα R	CTTAACATGTCCTGCGCTATCA			
C. perfringens β toxin (*cpb*)	CPβ F	TCAAACAACCCTGTATATGGAAATG	60	149	This study
	CPβ P	6FAM-ACGGAAGATATACTAATGTTCCTGCAACTG-TAMRA			
	CPβ R	GGAGCAGTTAGAACTACAGACAT			
C. perfringens β-2 toxin (*cpb2*)	CPβ2 F	TGCAACTTCAGGTTCAAGAGA	60	121	This study
	CPβ2 P	6FAM-ACCATTTGAGAAGCTTTAACATCATCTCCC-TAMRA			
	CPβ2 R	TTGTCTAGCAGAATCAGGGTTT			
C. perfringens enterotoxin (*cpe*)	CPe F	AGCTGCTGCTACAGAAAGATTA	60	101	This study
	CPe P	6FAM-CTGATGCATTAAACTCAAATCCAGCTGGT-TAMRA			
	CPe R	GAGTCCAAGGGTATGAGTTAGAAG			
C. perfringens ι toxin (*iap*)	CPia F	CGTGGAGGATATACCGCAAT	60	116	This study
	CPia P	6FAM-TGGTCCTTTAAATAATCCTAATCCA-TAMRA			
	CPia R	GGTGTGAGCTTTAATGCGTTT			
C. perfringens ε toxin (*etx*)	CP etx F	AGCTTTTCCTAGGGATGGTTA	58	112	(Messelhausser et al., 2007)
	CP etx R	AACTGCACTATAATTTCCTTTTCC			
C. difficile toxin B (*tcdB)*	CD tcdB F	GAAAGTCCAAGTTTACGCTCAAT	56	176	(van den Berg et al., 2006)
	CD tcdB P	6FAM-ACAGATGCAGCCAAAGTTGTTGAATT-TAMRA			
	CD tcdB R	GCTGCACCTAAACTTACACCA			

3.2.6 Illumina sequencing and sequence data analysis

All DNA samples were sent to University of Minnesota Genomics Center (Minneapolis, MN, USA) for Illumina sequencing using an Illumina MiSeq instrument. The V1-V3 regions of the 16S rRNA gene was amplified using primers Meta_V1_27F

(TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**AGAGTTTGATCMTGGCTCAG**)

and

Meta_V3_534R

(GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**ATTACCGCGGCTGCTGG**).

The bold part of each primer was complementary to the 16S sequence while upstream sequences corresponded to Illumina adapters required for sequencing and multiplexing. Paired-end sequencing was performed according to manufacturer's instructions.

QIIME pipeline (MacQIIME 1.8.0 20140103 OS10.6) (Caporaso et al., 2010) was used to analyze the sequences of 16S rRNA gene amplicons. PANDAseq (Masella et al., 2012) was used for quality filtering and assembly of the two ends of each read into contigs. Pairs with miscalled or uncalled bases in the overlapping region were discarded.

Operational Taxonomic Units (OTUs) were generated using the UPARSE workflow (Edgar, 2013). Briefly, all sequences were merged into a single file, and the library name was used for multiplexing. To minimize computing time, sequences were de-replicated and sorted by abundance. Singletons, which are unique read sequences in the data set, were discarded to improve specificity. Sequences were clustered into

OTUs by USEARCH (Edgar, 2010) using the Greengenes reference database, released in October 2013, and a 97% similarity threshold. UCHIME (Edgar, 2010; Edgar et al., 2011) was used for filtering of chimeric sequences. OTUs with abundance below 0.005% of the total number of sequences were discarded (Bokulich et al., 2013).

Downstream analyses including taxonomy assignments, and alpha and beta diversity estimations were conducted using the QIIME workflow core_diversity_analysis.py, with a sampling depth of 7939 (Navas-Molina et al., 2013). This analysis was conducted with default parameters: taxonomy was assigned using Ribosomal Database Project (RDP) Classifier V2 (Wang et al., 2007) , alpha diversity was estimated by Phylogenetic Diversity (PD) Whole Tree, Chao 1 and Observed Species (Colwell et al., 2012) indices, beta-diversity was estimated through UniFrac distances (Lozupone & Knight, 2005; Vázquez-Baeza et al., 2013).

3.2.7 Statistical methods

Data analysis of relative abundance and qPCR results were performed in Statistical Analysis System (SAS version 9.3; SAS Institute, 2012). The gene counts of Clostridial clusters I and XI were converted into percentage of total bacteria gene counts for analysis. Mixed Procedure (Proc MIXED) was used based on randomized completely block design with repeated measurement. In the model, diet and week as well as diet*week were considered as fixed effects, while block and pigs were considered as random effects. Comparisons of treatments were determined by contrast of target groups (SAS version 9.3). For relative abundance analysis, data from week 0 (at weaning) were used as covariate compared with the difference between combination groups to check the effects of *L. reuteri* and their metabolites (exopolysaccharides and reutericyclin).

Statistically significant differences were reported with $p<0.05$ after Bonferroni-adjusted. Normality of all variables was tested by Kolmogorov-Smirnoff test (Young, 1977). Results are presented as means with standard errors. Alpha- and beta-diversity were analyzed in MacQIIME v1.8.

3.3 Results

3.3.1 Characteristic of pigs

To investigate the development of fecal microbiota in weanling pigs, we analyzed the microbiota communities in 36 pigs obtained from an animal trial that tested the effects of EPS on ETEC in weaning pigs (Chapter 2). Paired-end reads with 300bp of each end resulted in sequences of 500bp targeting to the entire V1-V3 region of the 16S rRNA gene. A total of 137 fecal samples collected from three-week feeding trial were analyzed (see Table 3-1 for the experimental design). All of the pigs remained healthy during the experimental period and no diarrhea or any clinical signs of disease were observed.

3.3.2 Bacteria taxonomic structure: richness and diversity

In this study, a total of 5, 292,722 sequences with a minimum of 7939 sequences per sample was obtained after quality controls and was used for microbial communities analysis based on OTUs. A total of 7434 OTUs were obtained from reads with 97% pairwise-identity cut-off clustering. Greengenes database released in October 2013 was used as reference database to assign the OTU representative sequences into different bacterial taxonomic groups.

According to taxonomic analysis of the V1-V3 16S rRNA gene amplicon reads, a total of 7 phyla, 27 families, 42 genera and 49 species of bacteria were identified from the pig feces. The microbiota phyla in fecal samples included: *Bacteroidetes* (40.8%-

45.8%), *Firmicutes* (35.8%-45.1%), *Proteobacteria* (0.9%-12.9%), *Tenericutes* (0.7%-5.2%), *Spirochaetes* (1.4%-3.1%) and *Planctomycetes* (0%-1.3%) (Figure 3-3). Diversity analysis (Alpha and Beta diversity) results indicated that the effects of treatments were smaller and insignificant when compared with the differences occurring over time. In Alpha diversity metrics (within sample diversity), species richness of week 3 was significantly higher ($p<0.001$) than the other weeks (Figure 3-1) but no significant difference between diets was observed. All of the three phylogeny-based metrics (Phylogenetic Diversity Whole Tree, Chao 1 and Observed Species) implemented in QIIME v1.8 showed the same trend. Beta diversity results also showed strong groupings of fecal samples taken from the same week (time) and a statistically significant difference between weeks was observed ($p<0.0001$) (Figure 3-2). UniFrac distance metrics (Lozupone & Knight, 2005) indicated that the distance between samples within each week was always smaller than the between week comparisons and the PCoA analysis also showed that samples clustered together in each week (Figure 3-2).

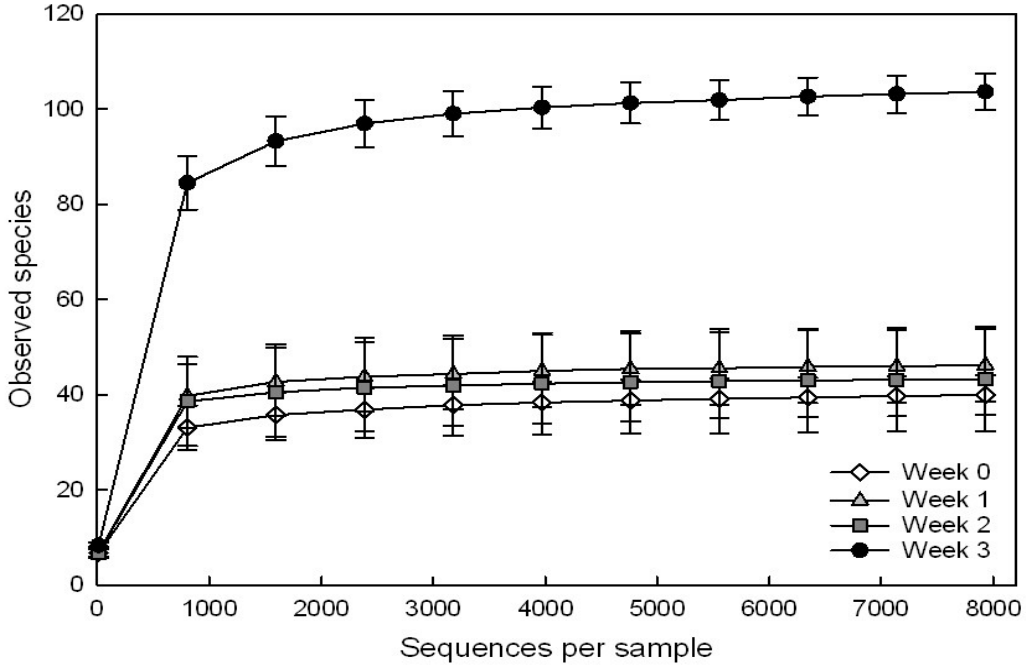

Figure 3-1. Number of observed species in fecal microbiota of pigs, determined by Illumina sequencing of 16S rRNA tags. Rarefaction curves for observed species were calculated in QIIME with the sample depth of 7939 sequences from 137 fecal samples obtained at week 0 (n=29), week 1 (n=36), week 2 (n=36) and week 3 (n=36) of weaning.

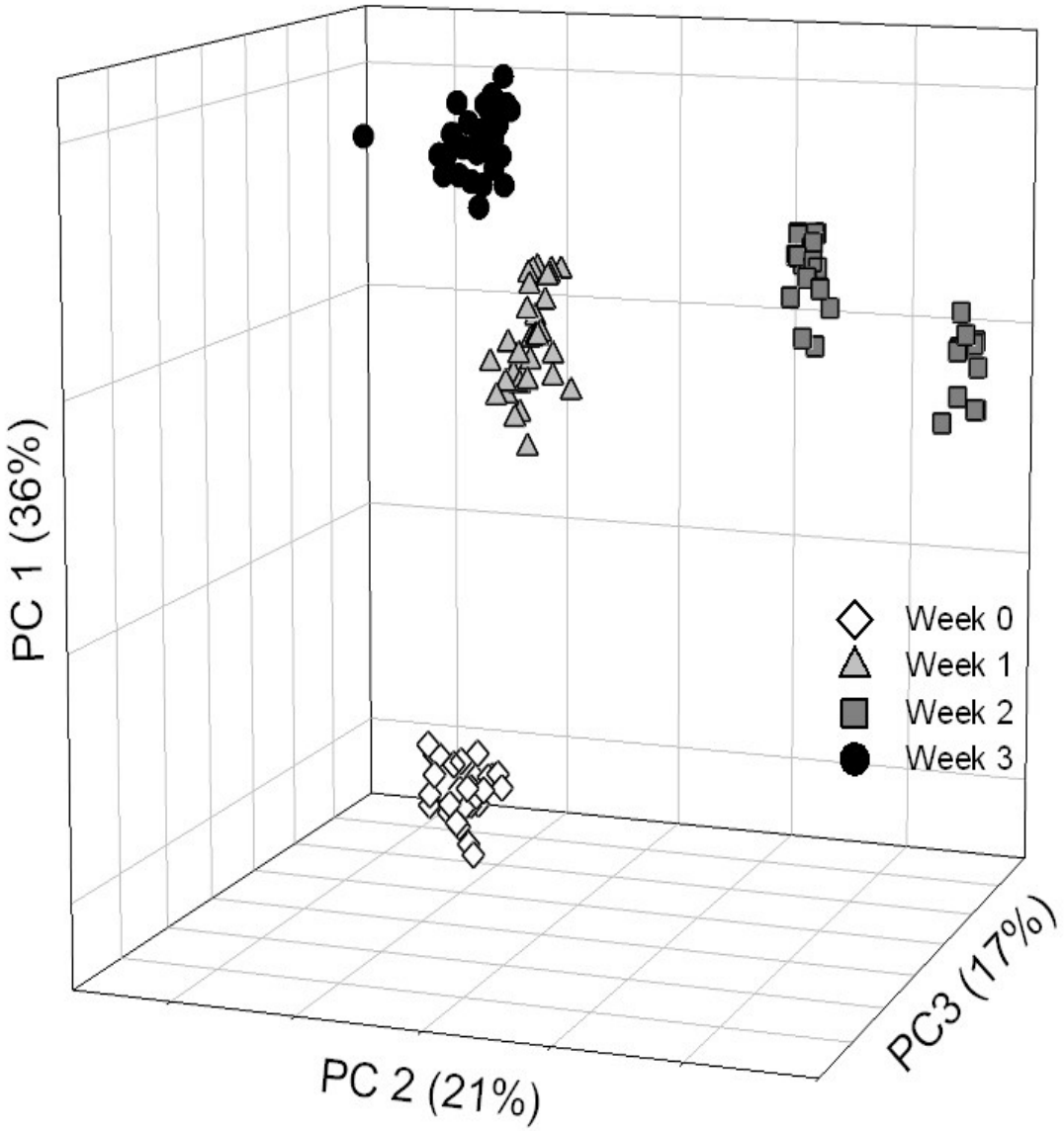

Figure 3-2. Separation of pig fecal microbiota by time. 3D plot showing unweighted UniFrac-based principle coordinate analysis (PCA). Each dot represents an individual sample collected at the start of weaning week 0 (n=29) or after week 1 (n=36), week 2 (n=36) and week 3 (n=36) of weaning.

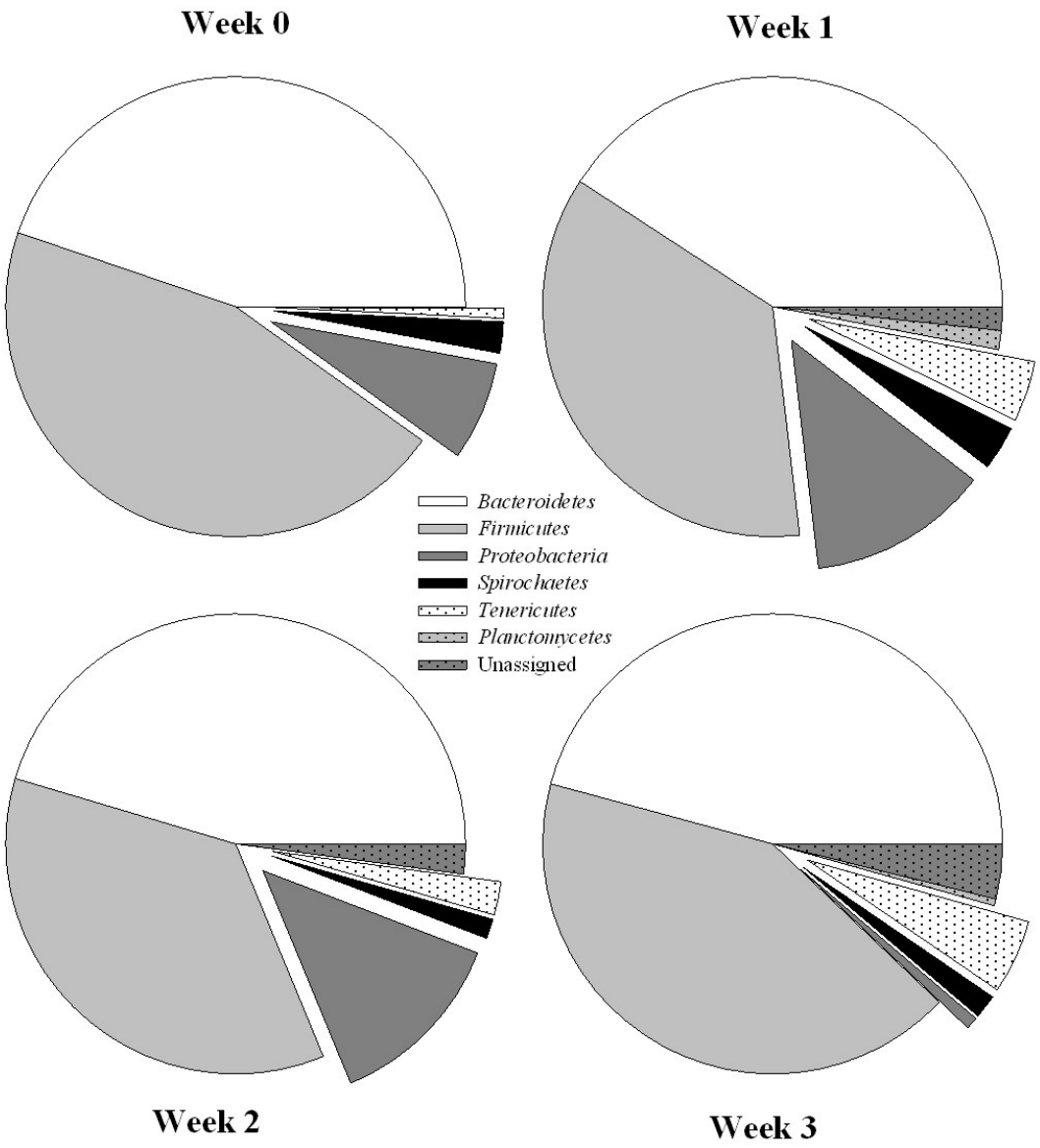

Figure 3-3. Microbiota composition at the phylum level in pigs at weaning (week 0) and one week (week 1), 2 weeks (week 2) and 3 weeks (week 3) after weaning. Data represent the median proportions of each phylum as determined by the RDP (Ribosomal Database Project) classifier.

3.3.3 Transition of bacteria over time and prevalent bacterial genera

To investigate the shift of fecal bacteria in weanling pigs during the study period, we analyzed the relative abundance of bacterial groups over time at the Family level

(Table 3-3). Changes within the phyla *Bacteroidetes* and *Firmicutes* were observed at this level. In phylum *Bacteroidetes*, *Prevotella* and genus *S24-7* increased while genus *Bacteroides* decreased. The reduction of *Ruminococcaceae* abundance corresponded to an increase of unassigned organisms and several genera in the phylum *Firmicutes*. The overall increase of microbial diversity could be mainly attributed to these changes. *Tenericutes* increased over time and *Proteobacteria* (unassigned *Enterobacteriaceae*) peaked at week 1 but decreased thereafter. *Planctomycetes* increased while there was no significant change of *Treponema* in phylum *Spirochaetes*. At weaning (week 0), genera from *Ruminococcaceae* (17.69%), *Bacteroides* (12.99%) and other *Bacteroidales* (10.74%) were the top three groups of bacteria. One week after weaning, *Lactobacillus*, as well as *Prevotella* and genus from family P-2534-18B5 and S24-7, became the dominant bacterial genera detected throughout the study period.

Table 3-3. Relative abundance (%) of bacterial groups in fecal microbiota of pigs at week 0 (at weaning) and weeks 1, 2, 3 (after weaning), determined by Illumina sequencing of 16S rRNA tags.

Genus	W0*	W1	W2	W3
Bacteroidetes				
Bacteroides	12.99±2.58	ND	ND	ND
Parabacteroides	2.19±4.32^{ab}	3.09±2.57^{a}	1.67±0.76^{b}	1.15±0.43^{b}
Prevotella	8.13±0.71^{a}	9.6±1.14^{ab}	14.36±1.03^{b}	20.19±2.79^{c}
[F: S24-7]	3.9±2.58^{a}	10.49±5.52^{b}	14.6±6.87^{c}	15.29±0.45^{c}
CF231	ND	ND	ND	0.47±0.39
[F: Paraprevotellaceae]	ND	2.33±1^{a}	0.63±0.07^{b}	1.31±0.7^{b}
Other (O: Bacteroidales)	10.74±22.44	ND	ND	ND
[O: Bacteroidales]	1.71±0.04^{a}	ND	1.71±0.04^{a}	3.17±1.54^{b}
[F: P-2534-18B5]	5.36±0.01^{a}	15.56±31.64^{b}	14.09±10.11^{b}	3.89±3.09^{a}
Firmicutes				
Lactobacillus	8.19±14.93^{a}	11.83±1.32^{ab}	14.96±5.1^{b}	11.47±10.86^{ab}
Other (O: Clostridiales)	ND	1.11±0.22^{a}	ND	0.24±0.04^{a}
[O: Clostridiales]	3.96±3.58^{ab}	4.37±10.61^{a}	1.81±1.7^{b}	6.46±4.14^{a}
[F: Christensenellaceae]	2.01±0.14^{a}	2.93±10.65^{a}	1.62±1.2^{a}	0.59±0.51^{a}
[F: Clostridiaceae]	ND	ND	ND	0.46±0.02
Blautia	ND	ND	ND	0.47±0.08
Coprococcus	ND	0.91±1.71^{a}	2.42±0.43^{a}	0.86±0.57^{a}
Lachnospira	ND	ND	1.06±0.16^{a}	0.82±0.07^{a}
Roseburia	1.3±1.72^{a}	ND	0.3±0.02^{b}	1.05±0.32^{a}
Other (F: Lachnospiraceae)	2.61±0.32^{a}	ND	0.47±0^{b}	ND
[F: Lachnospiraceae]	3.8±1.57^{a}	4.62±3.72^{a}	2.16±1.61^{b}	2.86±0.74^{ab}
Faecalibacterium	ND	ND	1.01±0.29^{a}	0.7±0.09^{b}
Oscillospira	1.38±0.89^{a}	ND	0.41±0.12^{b}	1.5±0.22^{a}
[F: Ruminococcaceae]	17.69±7.02^{a}	6.68±0.4^{b}	4.93±2.67^{b}	5.74±0.75^{b}
Dialister	ND	ND	ND	1.12±0.01
Megasphaera	ND	0.84±0.04^{a}	2.58±0.01^{ab}	3.73±0.03^{b}
Mitsuokella	ND	ND	ND	1.11±0
Bulleidia	ND	ND	1.1±0.08^{a}	0.84±0.11^{a}
Catenibacterium	ND	ND	0.52±0^{a}	0.46±0.06^{a}
Eubacterium	0.48±0.28^{a}	ND	ND	0.41±0.01^{a}
p-75-a5	2±1.33^{a}	1.64±1.45^{a}	2.01±0.92^{a}	1.07±0.66^{a}
Planctomycetes				
[F: Pirellulaceae]	ND	1.42±0.22^{a}	ND	0.5±0.68^{b}
Proteobacteria				
Desulfovibrio	1.36±0.32	ND	ND	ND
Succinivibrio	ND	3.07±0.27^{a}	ND	0.38±0.63^{b}
[F: Enterobacteriaceae]	4.95±2.65^{ab}	9.09±14.54^{a}	9±15.56^{a}	0.48±0.03^{b}
Spirochaetes				
Treponema	2.08±0.85^{ab}	3.54±1.22^{a}	1.6±5.36^{b}	1.86±0.52^{b}
Tenericutes				
[O: RF39]	1.13±0.26^{a}	4.7±2.83^{b}	2.25±1.42^{a}	5.38±1.13^{b}
Unassigned	ND	1.79±0.28^{a}	2.33±0.36^{a}	3.97±2.63^{b}
Total	**97.96**	**99.61**	**99.6**	**100**

- Data are presented as means and standard deviation.

- [abc] means for the relative abundance of the same taxon, data do not share the same letter indicates statistically significant difference ($P<0.05$). ND means not detected.
- Unassigned genera are presented with upper level of family (F) or order (O) in square brackets. "Unassigned" means a good hit to a particular sequence, but that sequence happens to have a poorly defined taxonomy itself at the genus level. "Other" means the assignment is ambiguous. (Greengenes Taxonomy released in October, 2013)
- * indicates that sample size of week 0 is 29.

3.3.4 Clostridial clusters and toxins quantified by qPCR

To determine the effects of reutericyclin on Clostridial clusters, Clostridial clusters I and XI and toxins were quantified using qPCR with specific primers and probes. The results are presented in Table 3-5 and Figure 3-4. From the qPCR data, both Clostridial clusters I and XI were detected in high gene counts in feces. *C. perfringens* toxins including β-, entero-, ι- and ε-toxin and *C. difficile* toxin B were all under the detection limit and only α- and β-2 toxins were detected. α- and β-2 toxins were mainly detected in samples collected at week 0 and in a few samples from week 1 (Table 3-5). Nonsignificant changes of Clostridial clusters and toxins between diet at each time point or within diets over time were observed.

3.3.5 Effects of treatments on bacteria species

To explore the effects of each diet on bacterial species, the relative abundance of each taxon was analyzed. Some differences in species in *Prevotella*, *Dialister* and *Mitsuokella* genera at week 3 after weaning (Table 3-4) were found, while no significant effect of diet on other was observed. Analysis of the relative abundance data using contrast for target comparisons was made for the following comparisons :

(1) *L. reuteri* TMW 1.656 (reutericyclin-positive) containing group (REU+ and REU-) vs. *L. reuteri* LTH 5794 (reutericyclin-negative) containing group (LEV+ and LEV-);

(2) Reutericyclin-positive group (REU+ and REU-) vs. the other four groups;

(3) EPS-positive group (REU+ and LEV+) vs. EPS-negative group (REU- and LEV-);

(4) EPS-positive group vs. the other four groups;

(5) Bacteria containing group (REU+, REU-, LEV+ and LEV-) vs. control and acidify groups.

Based on these comparisons, the EPS-positive group did not show a significant difference in all bacterial species compared with the EPS-negative group or the other four groups. Significant differences in the other comparisons were mainly observed at the genus level of phylum *Bacteroidetes*, *Firmicutes* and *Proteobacteria* at week 3 (Table 3-4). Differences between fermented and unfermented treatments were significant. Fermented diets had a greater abundance of *Clostridiales* species but lower abundance of *Enterobacteriaceae* and CF 23 species. Effects of reutericyclin, produced by *L. reuteri* TMW 1.656, were also noted. The reutericyclin-positive group (REU+ and REU-) had a greater abundance of other *Mitsuokella* species compared with *L. reuteri* LTH 5794 group (LEV+ and LEV-) or all other four groups (Table 3-4).

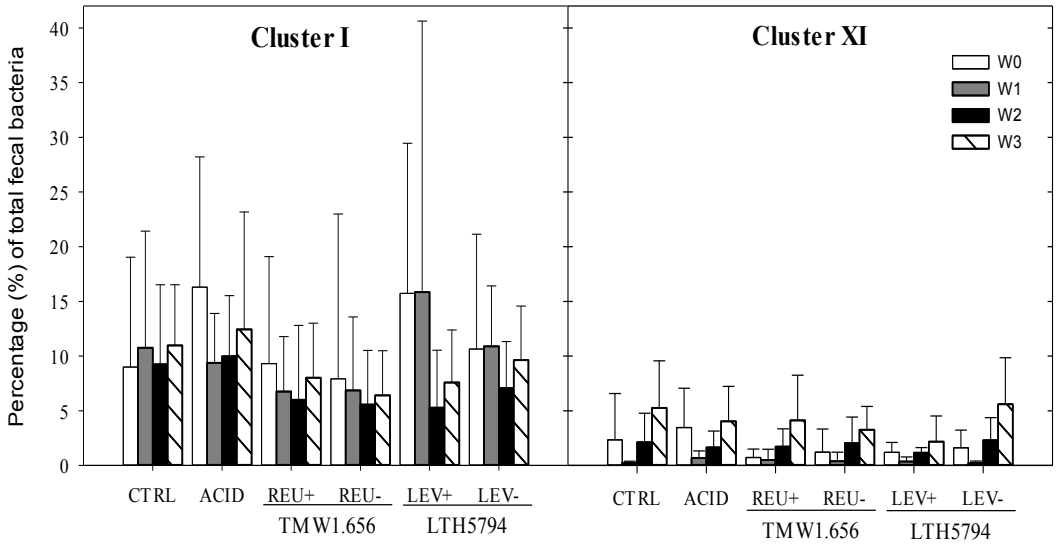

Figure 3-4. Percentage of *Clostridium* Cluster I and XI in total bacteria of pig feces collected at week 0 (W0, n=29), weeks 1 (W1, n=36), 2 (W2, n=36) and 3 (W3, n=36) of weaning quantified by qPCR. Data are presented as means, with their standard deviation represented by vertical bars. Raw data was provided by Xin Zhao.

Table 3-4. Effects of diets and reutericyclin (RTC) in fecal microbiota of pigs after 3 weeks of weaning (W3), determined by Illumina sequencing of 16S rRNA tags. Effects of diets were analyzed based on the relative abundance of bacteria groups. To analysing the effects of RTC, data from week 0 (at weaning) of each pig sample are used as covariate when P value was calculated. Only the significant effects are shown in the table.

Variables / Species	Effects of diets (relative abundance %)						Effects of RTC (P value)		
	CTRL	ACID	LEV+	LEV-	REU+	REU-	TMW1.656 vs LTH5479	RTC vs Others	Reuteri vs Non-reuteri
Bacteroidetes									
[G: Prevotella]	6.63±2.99^{B}	12.84±6.71^{A}	6.23±2.22^{B}	8.23±4.56^{AB}	6.06±2.58^{B}	5.05±3.3^{B}	0.68	0.27	0.07
Copri	10.42±8.44^{AB}	5.62±3.54^{A}	14.22±5.88^{AB}	8.59±6.46^{AB}	16.85±7.38^{B}	11.13±6.56^{AB}	0.87	0.46	0.16
[F: S24-7]	19.39±7.42	13.98±1.47	16.35±2.64	16.46±3.27	11.68±3.93	13.88±6.1	0.09	0.06	0.22
[G: CF231]	0.48±0.31	0.95±0.43	0.22±0.19	0.46±0.46	0.3±0.23	0.38±0.21	0.96	0.31	0.02
[F: p-2534-18B5]	5.18±4.91	2.5±1.56	4.16±3.66	3.3±3.59	3.47±6.01	4.72±6.03	<.0001	<.0001	<.0001
Firmicutes									
[O: Clostridiales]	4.96±2.08	4.26±1.48	6.68±2.67	8.89±3.96	6.76±2.53	7.22±3.88	0.64	0.47	0.02
[G: Coprococcus]	0.14±0.25	0.2±0.3	1.52±1.4	1.21±1.73	1.36±3.25	0.73±0.65	0.049	0.35	0.07
[G: Oscillospira]	2.58±1.08	1.4±0.45	1.46±0.45	1.29±0.38	1.16±0.69	1.08±0.51	0.88	0.43	0.03
[G: Dialister]	0.19±0.37^{A}	0.03±0.06^{A}	0.02±0.03^{A}	0.38±0.86^{A}	4.37±7.08^{B}	1.72±3.08^{AB}	0.10	0.06	0.20
Other (G: Mitsuokella)	0.35±0.35^{AB}	0.07±0.12^{A}	0.16±0.2^{A}	0.15±0.24^{A}	3.74±5.43^{C}	2.2±0.86^{BC}	0.0001	<.0001	0.01
Proteobacteria									
[F: Enterobacteriaceae]	1.22±1.45	0.89±1.11	0.42±0.59	0.05±0.05	0.21±0.42	0.09±0.13	0.83	0.21	0.01

Data indicate the effects of diets are presented as mean and standard deviation. ^{ABC} suggests that for the relative abundance of the same taxon, data with different letter indicates statistically significant difference. Data indicate the effects of RTC are presented as P value. *P<0.05* indicates statistically significant difference. ND means not detected. Unassigned species are presented as square brackets with upper level of genus (G), family (F) or order (O).

Table 3-5. Quantification of alpha and beta-2 toxins of C. perfringens in fecal samples collected at week 0 (W0), week 1 (W1), week 2 (W2) and week 3 (W3) of weaning pigs.

Variables / Diets	α toxin						β-2 toxin					
	W0		W1		W2	W3	W0		W1		W2	W3
	mean±SD	D/T	mean±SD	D/T			mean±SD	D/T	mean±SD	D/T		
CTRL	6.91±1.38	6/6	4.2±0.47	6/6	ND	ND	6.24±1.22	6/6	3.9±0.5	3/6	ND	ND
ACID	6.27±0.75	6/6	3.84±0.2	2/6	ND	ND	5.62±0.82	6/6	3.64	1/6	ND	ND
REU+	6.6±1.07	4/4	4.15±0.3	3/6	ND	ND	5.83±0.94	4/4	3.66±0.15	3/6	ND	ND
REU-	6.7±1.06	2/2	3.86±0.12	4/6	ND	ND	5.59±0.33	2/2	3.61	1/6	ND	ND
LEV+	6.99±0.41	5/5	4.16±0.3	4/6	ND	ND	6.27±0.34	5/5	3.82±0.1	2/6	ND	ND
LEV-	6.62±1.19	5/5	3.78±0.46	4/6	ND	ND	5.86±1.22	5/5	3.75±0	2/6	ND	ND

Data are presented as mean and standard deviation. ND means not detected. Unit are log gene copy number. D/T = samples detected / total samples collect. Data provided by Xin Zhao.

3.4 Discussion

The objective of this study was to investigate the effect of *L. reuteri* fermented diets and more specifically the effect of EPS synthesized by the two microorganisms on ETEC in the gut of weanling pigs. Feces were used as a surrogate. Microbiota communities were determined at weaning (week 0) and post-weaning (weeks 1, 2, 3). Both measures of microbiota diversity, alpha and beta diversity, did not alter significantly between treatment groups but indicated significant changes over time. No diarrhea or any clinical signs of disease were observed in any of the groups of pigs during the experimental study.

The development of human gut microbiota, especially establishment of the infant microbiota, have been well-studied by DNA sequencing in recent years (Solís et al., 2010; Dominguez-Bello et al., 2011). However, the equivalent development of bacteria in pigs is still poorly understood. The most important determinants of human gut microbiota during infancy involves delivery mode, type of infant feeding, antibiotics used by infant or mother and gestational age (Penders et al., 2006). In the first year of life, the infant microbial composition was characterized by low bacterial diversity and stability (Koenig et al., 2011). Compared with humans, newborn pigs are contacted with different microbes during or after birth and the microbiota was reported to remain stable after initial colonisation (Mathew et al., 1998). From birth to weaning, both human infants and piglets experienced a rapid ecological succession by *Lactobacillus* in the gut due to the consumption of milk (Tannock et al., 1990; Roger et al., 2010). The alteration of microbiota triggered by the change of diets from milk to solid food after weaning have not been extensively studied in either humans or pigs. Weaning usually does not cause major problems in infants because of the gradual adaptation period for the change of diet. However, weaning is a major issue in pigs.

The sudden change of diet, without adaptation, often causes diarrheal diseases resulting from the change in bacterial composition (Lallès et al., 2007). Thus it is important to monitor the bacterial alteration in pigs. This study is the first one to investigate and estimate the development of the pigs' microbiota at weaning.

Bacteroidetes and *Firmicutes* are the dominant phyla in pigs, accounting for up to 90% of the microbiota, which is in accordance with previous studies (Lamendella et al., 2011; Riboulet-Bisson et al., 2012a). In this study, *Lactobacillus* spp. and strict anaerobes such as *Ruminococcaceae, Bacteroides* and *Prevotella* were the dominant bacteria at week 0 (right after weaning), whereas after feeding the diets, *Ruminococcaceae* and *Bacteroides* decreased or were not detected but *Lactobacillus* and *Prevotella* spp. increased. This finding differed from the findings of Leser et al. (2002) that *Bacteroides* and *Prevotella* were the major components of Gram-negative bacteria in the porcine gastrointestinal tract. This difference could be attributed to the age and diets of the pigs as well as the sampling procedures. Leser et al. (2002) detected the large intestinal microbiota using growing pigs (individual weight 30 to 50 kg) fed a variety of diets while we investigated the fecal microbiota using weanling pigs (individual weight ≦20 kg) fed wheat diets.

Different diets result in a different bacterial composition. Pigs fed with green feeds had higher bacterial diversity than pigs fed with synthetic feeds in manures (Lu et al., 2014). Additional resistant starch in the diet can increase and stimulate the growth of Gram-negative bacteria in the colon of pigs (Durmic et al., 1998). Wheat used in the diets of this study, whether fermented or not, could contribute to the increase of *Prevotella*, which grows rapidly on starch media (Pitta et al., 2010). A significant increase in bacterial diversity was noted at week 3 and was attributed to several genera in the phylum *Firmicutes* and a large number of unassigned genera. The high

carbohydrate content of the wheat diets could be the reason, because significant diet-dependent reduction in *Firmicutes* levels was observed in fecal samples from obese individuals on low-carbohydrate diets (Duncan et al., 2008).

When compared with the effect of time, only a few significant changes in the intestinal microbiota were attributable to *L. reuteri* strains and their metabolites. *L. reuteri* is the only *Lactobacillus* species known to inhabit the gut of all vertebrates and mammals and it is believed to be a gut symbiont (Casas & Dobrogosz, 2000; Chen et al., 2014). It was reported to suppress the proliferation of enteric pathogens through its probiotic properties (Spinler et al., 2008). In line with the previous report (Spinler et al., 2008), a decrease of *Enterobacteriaceae* was associated with the fermented diets. This corresponded to our previous findings using qPCR as the analysis method that gene numbers of *E. coli* decreased significantly in groups fed fermented diets (Chapter 2). In contrast, the fecal microbiota were not influenced by the presence of EPS. This result would support that EPS used in this study have specific effects on ETEC but not the fecal microbiome (Wang et al., 2010; Chen et al., 2014).

In addition, the effect of reutericyclin secreted by *L. reuteri* TMW 1.656 was noted only in some taxons. Reutericyclin is active against Gram-positive bacteria such as *C. difficile* and *C. perfringens* (Hurdle et al., 2011; Cherian et al., 2014) and it was detected in high gene copy number in fecal samples by qPCR methods in this study (data not shown). Instead of observing the inhibitory effects of reutericyclin on Clostridia, the Illumina sequencing results demonstrated a significantly higher abundance of *Mitsuokella* (genus of *Clostridia*) in reutericyclin-positive group (REU+ and REU-). Both *Clostridium* cluster I and IV were detected in high counts by qPCR but significant difference between reutericyclin-positive and -negative groups were

not observed. The reason for this result is unclear because the production of reutericyclin in fermented feed was not quantified. The high gene counts detected by qPCR only indicated the presence of the gene but did not ensure the production or presence of reutericyclin. Thus, more research is necessary to investigate the particular effects of reutericyclin.

In conclusion, this study did not find many positive effects of *L. reuteri* metabolites on the composition of the fecal microbiota of weanling pigs but it did monitor the development of the bacterial community. In combination with the previous report (Chapter 2), the presence of EPS in the diet had specific effects on ETEC but did not alter the fecal microbial communities. The change of diets from milk to fermented wheat influenced the fecal microbiota. *Bacteroidetes* and *Firmicutes* are the dominant phyla in weanling pigs' feces. Bacterial diversity increased significantly within 2 weeks of weaning and this was mainly attributable to an increase of unassigned organisms and several genera in the phylum *Firmicutes*. This is the first in depth analysis of the development of pig microbiota after weaning. Results will support future efforts to manage the transition period in commercial pig production.

3.5 References

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4. General discussion and conclusions

4.1 Discussion

The studies present in this thesis introduce evidence that might influence either swine production or health care for infants. The potential of EPS to control post weaning diarrhea in pigs have been proven to be effective, which give a hint to prevent or control children diarrhea in developing countries. The six diets used in the feeding trial provide control vs the treatments and variable comparisons that ensure the reliability of the experimental design and the results.

PWD is a widespread and important disease in weaning pigs, causing financial loss in the swine industry. This thesis research compared the effects of two EPS produced by *L. reuteri* during fermentation and studied the potential application of reuteran-containing fermented feeds to control PWD in pigs. In addition, the development of fecal microbiota in weaning pigs was studied, which will provide information to help manage enteric bacterial infections during the weaning period.

Prebiotics, which are defined as a "selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health", have been widely studied in recent years (Gibson et al., 2004; Roberfroid, 2007). Prebiotics are non-digestible, fermentable carbohydrates that increase the population of *Lactobacillus* spp. in the gut and the diversity and stability of the microbiota in the colon (Konstantinov et al., 2004; Wellock et al., 2008). Dietary supplementation of inulin in 48 weaning pigs reduced the incidence and severity of post-weaning diarrhea but had no influence on pig performance after an experimental ETEC challenge (Nochta et al., 2009). Non-starch polysaccharides (NSP) can modulate the composition and

metabolic features of fecal miroflora with the effects being linked to individual NSP (Metzler-Zebeli et al., 2010). Bhandari et al. (2009) also reported that the use of raw potato starch did not affect growth performance but increased the prevalence of lactobacilli and improved fecal consistency scores in pigs, similar to those fed sub-therapeutic antibiotics. However, the low dose (7%) of raw potato starch resulted in more favorable results than the high dose (14%).

Unlike previous studies, the anti-adhesive glycans (EPS) used in this study have specific effects on ETEC with K88 fimbriae, which have competitive exclusion properties. Both *in vitro* (Wang et al., 2010) and *in vivo* (Chen et al., 2014) studies found positive effects of EPS against ETEC K88 strains. However, ETEC with K88 fimbriae were mainly found in infected newborn and suckling pigs, while in weanling pigs the infections were more related to ETEC with K99 fimbriae (Fairbrother et al., 2005). In the present study, the fact that the effect of EPS on ETEC was not significant compared with the EPS-negative group could be result from the ETEC K99 in the weanling pigs, further supporting the specific effects of EPS. This thesis also introduces a promising way for controlling children diarrhea in developing countries. Future studies can focus on the EPS that have effects on human linage ETEC and then given to human beings via food fermentation to prevent or control the diarrhea diseases in infants and children.

LAB involving fermented feed used in swine production has been previously studied (Lindgren & Dobrogosz, 1990; Olstorpe et al., 2008). Wheat fermentation by *L. reuteri* with added sucrose is a cost-effective way to include specific quantities of *in situ*-produced EPS without purification (Franz & Feuerstein, 1997; Welman, 2009). It is time-consuming and expensive to isolate and purify the EPS from LAB because of the necessary steps to remove the mono-, di- and oligosaccharides (Wang et al., 2010).

From the fermentation results in this study, the yield of reuteran and levan can be up to 5.6 ± 1.0 g/kg and 3.2 ± 0.6 g/kg of dough, respectively. Without purification, the *in situ* production of EPS in fermented wheat can be fed directly to the pigs. This is a cost-effective way for the swine industry to improve weanling pig health.

This promising feed can also be used in sows' diets to promote the health benefits to newborn pigs and suckling pigs. It was reported that the immune response in pregnant sows was stimulated by probiotics before parturition (Martín et al., 2009). When fed to sows before farrowing or during lactation or to newborn piglets, the number of pathogens present in the sow and piglet feces was decreased, contributing to the reduction of digestive disturbances and mortality (Danek and Novak, 1992; Martin et al., 2009). Clostridial enteric infections in newborn or neonatal piglets have shown to be transmitted from sows (Kohler et al., 1979). The fermented diets in this study indicated that fermented feeds, especially feed fermented with *L. reuteri* TMW 1.656, have the potential to reduce the presence of clostridia in weanling pigs. Thus, if the sows are fed these fermented diets, the clostridia present in sows could possibly be controlled and this could limit the transfer of clostridia to the piglets. However, research is needed to study these proposed effects. Unexplainably, the current study found reutericyclin had a positive effect on number of clostridia versus an inhibiting effect. Studies with gene mutant strains that cannot produce reutericyclin are required to unveil the contradictory findings.

In addition, the specific effects of EPS will limit its application in swine industry and also not able to expand to other animal species. Though the non-infection model closely matches commercial applications, the model did not provide conclusive evidence related to the protection against pathogens because all pigs remained healthy.

An infection model is required to convincingly demonstrate the specific effect of feed fermentation with EPS-producing lactobacilli against ETEC.

4.2 Conclusions

In this thesis, feed fermented by *L. reuteri* with the combination of metabolites showed beneficial effects on pig health. These results gave insight to the possible development of comparable applications in other farm animals and humans. The details of the composition of the microbiota of weanling pigs during the three weeks of weaning provide information to manage enteric infections. Overall, the results obtained from this research provide a promising way to manage potential enteric infections and to improve the health of pigs. Future research is required to focus on the specific effects of the microbial metabolites and the potential to utilize the beneficial effects of LAB fermented feed.

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