Long-term Impacts of Early Life Antibiotic Exposure on Intestinal Microbiota and Metabolic Disease

by

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Abstract

Although genetic factors, diet choices and sedentary lifestyle have long been considered main risk contributors for obesity and diabetes mellitus, years of study show that these factors cannot fully explain the increasing rate of worldwide metabolic disease. Epidemiological studies in recent years have correlated antibiotic exposure in early life with long-lasting metabolic consequences, particularly central obesity. Experimental evidence for this relationship has not been provided, and the cellular and molecular mechanisms are not clearly defined. The overall objective of this study was to explore the association between early life antibiotic treatment and modified programming of gastrointestinal tract and pancreas development that predispose to metabolic disease. A piglet model for the human infant was developed by administering therapeutic doses of amoxicillin to newborn piglets through the first 2 postnatal weeks and examining the immediate and long-term consequences of the treatment. Changes of microbial composition and microbial product sensing were examined during the period of this study. Likewise, glucose regulation, pancreatic development and gene expression levels to the related pathways were assessed. Study 1 demonstrated that early life antibiotic treatment altered pancreatic development, β-cell function, and modestly impaired glucose tolerance. Shifted microbial composition to a specific direction, alteration of SCFA metabolism in large intestine and LPS activities in circulation further confirmed our hypothesis that early exposure to antibiotics may programme pancreatic and intestinal development via microbial metabolites that play a causal role in metabolic disorder pathogenesis. This is the first

experimental evidence linking therapeutic antibiotics in the early life period to islet development and function later in life. The observed variations *in vivo* and in isolated islets support the epidemiological evidence and indicate further study on the relationship between early life microbiota and long-term metabolic status is warranted. In addition, as metabolic disease is usually triggered by multiple environmental factors and the outcomes of some programming effects will only show in certain conditions, a westernized diet or high fat diet is likely to faster or exacerbate the outcomes of antibiotic programming effects. Therefore, study 2 evaluated the suitability of pig as a model of high fat diet induced metabolic syndrome. This pig model developed central obesity and impaired glucose control during oral glucose tolerance test and secreted less plasma insulin during the test within 5 weeks of high fat feeding. This study provides important experimental evidence supporting epidemiological studies relating early life antibiotics and metabolic outcomes, as well as a metabolic challenge model to support future research.

Preface

This thesis is an original work by Jiaying Li. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Early life antibiotics and metabolic disease”, No. AUP00000922, 12/11/2013.

Dedication

Dedicated
To
My beloved
Parents

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taking care of the animals, offering help during OGTT test and providing invaluable suggestions for improving study procedures.

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List of Abbreviations

CON: Control group

ANTI: Antibiotic group

PND: Postnatal day

AUC: Area under the curve

BW: Body weight

GLUT: Glucose transport

OGTT: Oral glucose tolerance test

GSIS: Glucose stimulated insulin secretion

SEM: standard error of the mean

LPS: Lipopolysaccharide

IAP: Intestinal alkaline phosphatase

qRT-PCR: Quantitative real time- polymerase chain reaction

SCFA: Short chain fatty acids

HFD: High fat diet

LFD: Low fat diet

TLRs: Toll-like receptors

ADFI: Average daily feed intake

ADG: Average daily gain

- Chapter 1: Literature Review

1.0 Introduction

The worldwide prevalence of obesity and related diseases are increasing rapidly, and has become one of the greatest public health c[1]oncerns in our century (Swinburn et al., 2011; van Vliet-Ostaptchouk et al., 2014; Gupta et al., 2012). According to Statistics Canada, Canadian Health Measures Survey (CHMS) from 2009 to 2011 indicated that 22% of Canadians aged 18 to 79 had metabolic syndrome. In the United States, the situation is even worse. Overall prevalence of the metabolic syndrome has substantially increased from 15% in 1970s to 35% in 2011-2012 (Aguilar et al., 2015). Similar patterns were observed worldwide and were shown to be comparable across different ages (Finucane et al., 2011). Childhood obesity has more than doubled in children and adolescents in the past 30 years, which promotes higher increasing rate of obesity in adults (Ogden et al., 2014; National Center for Health Statistics, 2012). Metabolic syndrome is a collection of interrelated risk factors for cardiovascular diseases and diabetes, including dysglycemia, insulin resistance, hypertension, dyslipidemia, obesity and a low grade systemic inflammation (Alberti et al., 2009; Ahonen et al., 2012). Among these, central obesity is the major contributor that triggers the adverse metabolic effects. Obesity is thought to be caused by an underlying imbalance of energy expenditure and storage (Hill et al., 2012).

The exact mechanisms of the complex pathways of metabolic syndrome are still under investigation. The most important factors for the pathophysiology are genetics, high energy diet consumption and sedentary lifestyles (Pollex et al., 2006; Golay et al., 1997; Lakka et al., 2012). However, these factors cannot fully explain the increasing prevalence of metabolic syndrome. One emerging factor noted is the role of intestinal

[1] Part of Chapter 1.2 will be published in the following book.
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microbial populations in modulating numerous physiological processes in the host. The evidence is that both genetic and diet-induced obesity is associated with microbial dysbiosis (Brown et al., 2012). In human studies, the microbial community differs between lean and obese individuals (Ley et al., 2006). A difference in the composition of gut flora was also observed in genetically obese (*ob/ob*) and lean mice (Ley et al., 2005). Germ-free animals without gut microbiota colonization usually do not develop diet-induced obesity and antibiotic treatment that creates pseudo-germ-free mice community reduces adiposity and improves glucose metabolic outcomes of obese mice, indicating that the gut microbiota is essential for this process (Tremaroli et al., 2012). In addition, the obese and lean phenotypes are transferable. Germ-free mice that received microbiota from obese mice also developed obesity (Turnbaugh et al., 2006). Gut microbiota transferred from lean donors increased insulin sensitivity in individuals with metabolic syndrome (Turnbaugh et al., 2006, Vrieze et al., 2012). Manipulation of gut microbiota by prebiotics and probiotics administration has been shown to alter gut microbial community and improve metabolic parameters in genetic and diet-induced obese mice (Everard et al., 2011). Targeting gut microbiota in the obese individuals by modulation of prebiotics and probiotics has been widely explored. However, the molecular mechanisms driving the various effects of probiotics and prebiotics leading to a healthy gut are slowly emerging (Sanders, 2008; Delzenne et al., 2011). A considerable number of studies demonstrated that microbiota shifted after gastric bypass and was associated with reduced host weight and adiposity (Liou et al., 2013; Zhang et al., 2009). Obese individuals that receive microbiota from lean donors have significantly improved insulin sensitivity over a 6-week period (Vrieze et al., 2012). All these results from experimental manipulations of the gut microbiota suggest the involvement of microbiota in metabolic syndrome development, either as a consequence or a cause of obesity.

1.1 Intestinal Microbiota and Early Life Perturbation

1.1.1 Establishment of Commensal Microbiota in the GIT

Our understanding of the composition and function of the human gut flora has improved dramatically in recent years. The human GI tract is home to a diverse and complex microbial community that plays a central role in host homeostasis (Tremaroli et al., 2012). The whole gastrointestinal lumen harbours more than 100 trillion commensal bacteria, classified into 500-1000 species, which possess 100-fold more genes than human genome. Bacteria constitute the vast majority of the human gut microbiota, with the present of archaea, viruses and protozoa that interact through predation, competition and mutualism (Parfrey et al., 2011; Ley et al., 2006; Qin et al., 2010). Along the GI tract, colon is the location where microorganisms are the most abundant with 10^{11} to 10^{12} cells per gram (O'Hara et al., 2004.). Upon delivery, neonates leave a relatively sterile environment and are rapidly exposed to and colonized with diverse organisms from the environment (Matamoros et al., 2013). Although there have been over 50 bacterial phyla described in human GI tract, the majority of microorganisms in the human gut belong to the phyla of *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*, with respectively lower numbers of other phyla. This is thought to be attributable to the coevolution between the host and its commensal bacteria (Schloss et al., 2004; Harris et al., 2012). Although the microbiota is similar at the phylum level, the relative quantities of each species differ, indicating that the gut microbial population is unique to each individual (Lozupone et al., 2012). Microbial population differs along the GI tract. Many members of *Bacteroidetes* and *Firmicutes* are able to utilize host-indigestible polysaccharides and thus dominate in the colon, whereas *Proteobacteria* and some members of *Firmicutes* such as Lactobacillales are dominant in the small intestine with high levels of nutrients such as mono- and disaccharides (Koropatkin et al., 2012). The earliest colonizers are generally facultative anaerobes such as *Escherichia coli* and other Enterobacteriaceae, followed by strict anaerobes as *Bacteroides*, *Clostridium* and

Bifidobacterium spp (O'Toole et al., 2010). The composition and diversity are relatively unstable during the early postnatal period. Infant gut microbiota converged toward a more adult community structure in 2-5 years, when the microbiota of infants fully resembles that of an adult in terms of composition and diversity as children are exposed to a new milieu that includes solid food and individuals with diverse microbiota (Rodríguez et al., 2015; Palmer et al., 2007).

1.1.2 Primary Functions of Commensal Microbiota

Gut microbiota has evolved with humans as a mutualistic partner. Once established, gut microbiota fulfill a multitude of useful functions in metabolic, nutritional, physiological and immunological processes in the host. It exerts essential metabolic activities by fermenting non-digestible carbohydrates and providing energy to the colonocytes. These metabolic activities lead to the production of short-chain fatty acids (SCFA), vitamins and amino acids with the enzymes that the host lacks (Jeffery et al., 2013). Furthermore, a healthy gut microbiota is actively involved in protecting the host from invasion of pathogenic microorganisms by colonization resistance and releasing antimicrobial compounds (Kamada et al., 2013). The residential bacteria can also positively control the intestinal epithelial cell differentiation and proliferation through microbial metabolites and maintain mucosal barrier function (Round et al., 2009). Additionally, gut microbiota is vital for the postnatal development of innate and adaptive immune system (Tremaroli et al., 2012). The immune system is educated by the resident microbiota and becomes more favorable to their survival, which is mainly dependent on the early exposure of neonatal gut to lipopolysaccharides (LPS) and antigens from non-pathogenic commensal bacterial (Goldwater, 2015). Therefore, the immune system becomes appropriately tolerant to non-harmful bacteria while recognizing invading pathogens and taking responses once under attack. It has been shown that colonization of germ-free mice with gut microbiota resulted in an increase in immunological capacity (Round et al., 2009; Tlaskalová-Hogenová et al., 2011).

Microbial colonization of the infant GI tract is an essential process in the human life cycle. *In utero* and early postnatal period are particularly important, during which the host immune system and pancreas are still developing and disruptions of the developmental processes have been shown to induce long-term health problems (Russell et al., 2012; Portha et al., 2011).

1.1.3 Factors Influencing Infant Microbiota Development

Several factors are known to influence early microbial colonization including gestational age, mode of delivery, breast milk or formula and antibiotic exposure (Rodríguez et al., 2015). In addition to metabolic disease, it has been suggested that disruption of the gut microbiota is related to the pathological intestinal conditions such as Non-alcoholic fatty liver disease (NAFLD), colorectal cancer, inflammatory bowel disease (IBD) encompassing both ulcerative colitis (UC) and Crohn's disease (CD) (Henao-Mejia et al., 2012; Sobhani et al., 2011; Tamboli et al., 2004; Joossens et al., 2011).

Gestation time strongly influences the subsequent colonization of the infant microbiota. Preterm and full-term infants significantly differed in gut microbiota composition with more pathogenic bacteria found in preterm infants and higher proportion of *Bifidobacterium*, *Lactobacillus* and *Streptococcus* present in full-term infants (Arboleya et al., 2012). A substantial difference in patterns of early microbial colonization has been observed in infants born through maternal birth canal as compared to cesarean section (Mueller et al., 2015). It was demonstrated that the early bacterial populations were mainly structured by the type of microbes that the child encountered at birth. Early colonizers in neonates by C-section resemble microbes in the hospital environment or mother's skin, while microbiota in naturally born babies is more similar to the mother's vaginal microbiota (Dominguez-Bello et al., 2010). The different pattern in C-section babies is related to impaired development of the

immune system and increased risk of obesity, IBD and asthma (Cho et al., 2013; Huh et al., 2011; Bager et al., 2012; van Nimwegen et al., 2011). Apart from delivery mode, infant intestinal microbiota is also influenced by the mode of feeding. *Streptococci* and *Staphylococci* pass through breast milk are among the earliest colonizers of the infant intestine (Hunt et al., 2011). Breastfed infants have been shown to carry a more stable and uniform population and contain significantly higher percentage of *Bifidobacterium* and *Lactobacillus*, which are some of the most well-known probiotics that promote gut health (Fernández et al., 2013). The beneficial effects of breast-feeding involve protecting against the development of diarrhea and necrotizing enterocolitis in newborns, autoimmune disease particularly coeliac disease and metabolic syndrome in childhood and adults (Guaraldi et al., 2012).

Notably, antibiotic exposure early in life has consistently been shown to change the gut microbiome in humans and animals and impacts the development of long-term immune-mediated diseases and metabolic diseases (Russell et al., 2012; Zeissig et al., 2014). Broad-spectrum antibiotics are frequently prescribed to infants to protect from infections (Trasande et al., 2013). In 2010, a broad-spectrum antibiotic amoxicillin was the top of the list of antibiotics used for pregnant women (Mitchell et al., 2011). It is notable that an epidemiological study spanning 2001-2013 revealed that early exposure to broad-spectrum antibiotics was associated with obesity, while narrow-spectrum drugs were not at any age or frequency (Bailey et al., 2014). In addition to enhancing antibiotic resistance among populations, antibiotic overuse can disrupt the overall microbial ecology. Although the gut microbiota is rather resilient to disruptive factors, microbial ecology can be severely altered if exposed too early in the development period (Fouhy et al., 2012). An example of antibiotic-induced disruption is the overgrowth of enteric *Clostridium difficile,* which benefits from the ecological disruption (van Nood et al., 2013). It has been shown that the antibiotic-induced health issues are caused by the dysbiosis following antibiotic exposure, rather than antibiotic itself. Germ-free mice receiving microbiota from

antibiotic treated mice recapitulate the metabolic results. Despite short-term exposure to antibiotics early in life, long-term phenotypes related to metabolic syndrome showed up later in life (Zeissig et al., 2014; Cox et al., 2014). In humans, administration of antibiotics during the first six months of life leads to a significant increase in body weight in children. However, antibiotic exposure later in infancy did not show this weight-promoting effect, suggesting that there might be a critical window of opportunity in obesity as well (Ajslev et al., 2011; Trasande et al., 2013). It has been demonstrated that there was inter-individual variation in response to the antibiotic treatment when three healthy individuals were exposed to the same type of antibiotic, indicating involvement of genetic or environmental factors in determining the host metabolic responses. The limitation in human studies makes animal models more attractive for studying microbial influence when both genetic and environmental factors are known, easily controlled and manipulated (Dethlefsen et al., 2008).

1.2 Microbiota-related Modulation of Metabolic Processes in the Host

1.2.0 Introduction

In considering how microbes affect our metabolic processes, it is important to consider how we define metabolism. Metabolism encompasses the life supporting biochemical transformation of molecules within and between cells that are required for life. These processes are made possible by enzymes that catalyze reactions and are coordinated through diverse signaling molecules and hormones (Tremaroli et al., 2012). Microbes both contribute to these biochemical processes by providing and modifying nutrients, as well as by modifying how the host processes and allocates nutrients (Nicholson et al., 2012).

Mammals utilize a complex set of regulatory processes mechanisms to maintain

equilibrium, and have evolved to include microbial contributions to this process. For example, bile acid metabolism is highly influenced by microbial bile acid deconjugation (Sayin et al., 2013). We also have receptors for molecules that are generated through microbial fermentation (e.g. butyrate) (Rasoamanana et al., 2012). Therefore, alterations in the gut microbiota, and how these contribute to metabolic regulation can disrupt metabolic homeostasis, resulting in the development of metabolic disease. The processes in the body impacted by the microbiota include many aspects key to metabolic outcomes including regulation of food intake, deposition and mobilization of fat, insulin secretion and glucose uptake (Guilloteau et al., 2010; Tremaroli et al., 2012).

It has long been recognized that the intestinal microbiota increases the host's ability to derive nutrients from the diet. The most obvious example is through the digestion of dietary fibre, as mammals lack the enzymes required to digest these non-starch polysaccharides. This increased provision of energy can both contribute to obesity development and promote health. As will be discussed later in the chapter an expansive effort continues to explore this relationship. In 2006 the research team led by Dr. Jeffrey Gordon was the first to demonstrate that not only presence, but also the composition of intestinal microbes govern the development of obesity, suggesting that there was an opportunity to treat obesity by modifying our microbes. They showed that by transplanting the microbiota of an obese mouse into a lean mouse, an obese phenotype could be imparted (Turnbaugh et al., 2006). This discovery created great excitement in the research community and spurred efforts to understand the mechanisms through which microbes could modulate host metabolic processes to result in beneficial and adverse outcomes. In this chapter we will discuss how microbes contribute to the modulation of metabolic processes in the body, discussing the workings of this normal process and how disruption of these pathways can contribute to adverse outcomes such as cardiovascular disease, obesity and diabetes.

1.2.1 SCFA Metabolism

Humans and other vertebrates have a very limited amount of glycoside hydrolases to degrade the bulk of complex dietary fiber. In the distal gut, the partially and non-digestible polysaccharides that have not been absorbed by the host in the upper gastrointestinal tract are fermented to SCFA (Flint et al., 2008). The microbial community produces SCFA as necessary waste products to maintain redox equivalent production and balance pH in the intestinal lumen (van Hoek et al., 2012). SCFA are a subset of fatty acids with less than 6 carbon atoms of which acetate (C2), propionate (C3) and butyrate (C4) are the most abundant (Cook et al., 1998). Even though SCFA provide a relatively small amount of energy (approximately 5-10%) for healthy people, they make a significant contribution to the body's daily energy requirements and also have profound effects on host processes, including energy utilization, host-microbe signaling, and control of pH in the colon, which affects microbial composition, gut motility and epithelial proliferation (Musso et al., 2011). In humans, the production rate of acetate, propionate and butyrate in the colon is in a molar ratio of around 60:25:15 (Tazoe et al., 2008). In healthy people, 95% of SCFA are absorbed rapidly while the remaining 5% are excreted in faeces. Butyrate is mostly utilized by colonic epithelial cells, where it is converted to ketone bodies or oxidized to carbon dioxide (Louis et al., 2007), whereas propionate and acetate reach the liver through the portal vein. Propionate is utilized largely for gluconeogenesis in the liver, while acetate either remains in the liver or is released to the peripheral tissues as a substrate for lipogenesis and cholesterol synthesis (Wolever et al., 1991, Vernay, 1987, Samuel et al., 2008). In the gut, SCFA are transported across the apical and basolateral membranes of colonocytes through passive diffusion of undissociated SCFA and active transport of dissociated SCFA anions (Sellin, 1999).

1.2.2 SCFA-Energy Source

It has been shown that either supplementation of butyrate in the diet or oral

administration of acetate to rodents can protect against both genetic or diet induced obesity, independent of food intake suppression. This is associated with an overall increased energy provision to the host (Lin, Frassetto et al. 2012). However, as an energy source that can contribute to weight gain, how increased microbial SCFA, through the provision of fiber, can paradoxically reduce obesity continues to be a major area of investigation. The hypothesis is that the effects of SCFA on obesity may depend on the balance between their role as energy source and their role in regulating gene expression and release of gut hormones to inhibit satiety and regulate metabolic processes.

In germ-free mice, Bäckhed and his colleagues performed a series of experiments to show the influence of microbiota on adiposity. First, they noticed 42% less fat in germ-free mice, which have very low SCFA production compared with conventionally raised mice even with higher food intake. Conventionalization of germ-free mice yielded a 57% increase in total body fat and increase in insulin resistance while these mice showed lower chow consumption compared with germ-free mice, suggesting better energy harvest and storage ability (Backhed, Ding et al. 2004). Previous studies showed that germ-free rodents excreted twice as many urinary and faecal calories as conventional rats fed the same diet due to the inability of germ-free mice to ferment dietary polysaccharides into SCFA, which account for the low body weight in germ-free mice (Wostmann, Larkin et al. 1983; Hoverstad and Midtvedt 1986). In another study, leptin-deficient *ob/ob* mice showed higher concentration of caecal SCFA than the wild-type mice, more caloric extraction from the diet and reduced energy content in their faeces, suggesting that gut microbiota enhances adiposity through increasing energy extraction from diet and by modulating fat storage. The obesity phenotype was transmissible through faecal transplants from obese to germ-free mice (Turnbaugh, Ley et al. 2006). This has also been extended to human microbiota populations by introducing faecal microbes from discordant obese twins to germ-free mice. Germ-free mice colonized with obese faeces gained more

weight and adiposity, however inconsistent with other studies showed lower concentration of caecal propionate and butyrate. When obese mice received a healthy diet (low-fat and high fiber) and were cohoused with mice containing lean microbiota, the lean microbiota finally dominated in the obese mice and prevented increased adiposity (Ridaura, Faith et al. 2013). They also showed that obese microbiota was not able to colonize lean mice when fed with low-fat diet. The fact that these mice remained lean suggests that diet is the prominent factor deciding which phenotype develops.

Several studies have shown that obese people have higher levels of faecal SCFA than lean, but until recently it was unclear whether this was due to increased SCFA production or reduced absorption, and whether the *Firmicutes* and *Bacteroidetes* ratio is related to faecal SCFA concentration remains inconsistent (Schwiertz, Taras et al. 2010; Patil, Dhotre et al. 2012; Teixeira, Grzeskowiak et al. 2013; Fernandes, Su et al. 2014). Rahat-Rozenbloom and his colleagues confirmed the hypothesis that higher faecal SCFA in overweight and obese people is not due to the differences in SCFA absorption or diet but SCFA production (Rahat-Rozenbloom, Fernandes et al. 2014). In this study, dialysis bags with a SCFA solution were used to measure the SCFA absorption; calculated by the SCFA disappearance from the dialysis bag divided by the SCFA present at the baseline. The absorption rate was the same in obese and lean individuals, and with a similar dietary intake, they further concluded that it is due to the higher production of SCFA by colonic microbiota in obese people than in lean individuals (Rahat-Rozenbloom, Fernandes et al. 2014). Further studies are needed to reveal the underlying mechanisms of how the higher SCFA production contributes to adiposity and obesity.

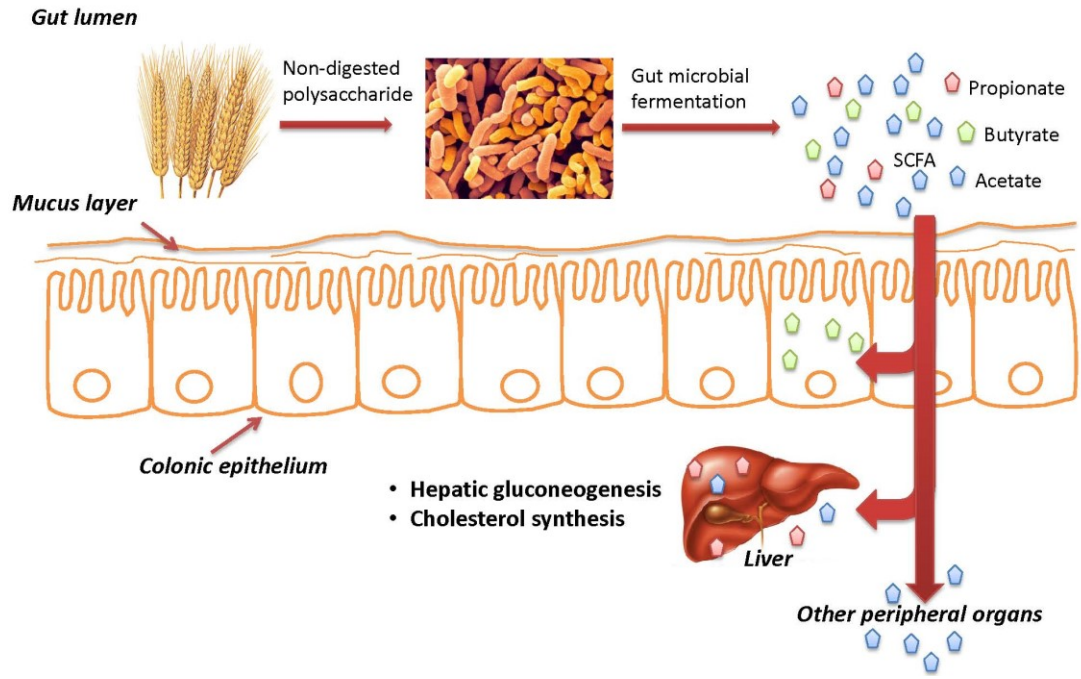

Figure 1.1 Summary of short chain fatty acids (SCFA) function as energy source. In the large intestine, gut microbiota ferment nondigestible polysaccharides to SCFA, including acetate, butyrate and propionate, which provide 5-10% energy for healthy humans. Once absorbed by intestine epithelium, butyrate is rapidly used by colonocytes, while propionate is used for hepatic gluconeogenesis and acetate is used for lipogenesis and cholesterol synthesis in liver and other peripheral organs.

1.2.3 SCFA-Sensing and Signal Transduction

In addition to providing energy, SCFA enter the bloodstream and act as signaling molecules. SCFA modulate biological responses of the host largely through two major mechanisms. The first involves epigenetic modification of DNA and histones, which directly regulate gene expression. The second is via G protein-coupled receptor (GPR) activation sending signals to cells leading to a cascade of metabolic changes.

1.2.3.1 Epigenetic

SCFA have a number of effects on cells, many of which are mediated through inhibition of histone deacetylases (HDAC). HDAC regulate gene transcription associated with pathologic processes by compacting chromatin and making it less accessible to transcriptional activators. Butyrate is well known as an inhibitor of HDAC, while propionate is less effective and acetate is completely inactive in colon cancer cell lines(Waldecker, Kautenburger et al. 2008). However, the impact of SCFA is also dependent on tissue type. Oral administration of acetate has been shown to inhibit both HDAC2 activity and protein expression in the rodent brain (Soliman and Rosenberger 2011). Inhibition of HDAC activity is the main mechanism through which butyrate affects the expression of proinflammatory cytokines in humans(Zeng, Lazarova et al. 2014). HDACs are involved in the pathogenesis of diabetes and are currently of interest as targets for the treatment of the disease (Christensen, Dahllof et al. 2011). In addition to epigenetically modifying histone acetylation profiles, Remely et al. recently demonstrated that SCFA could regulate methylation of *GPR41* in obese and Type 2 diabetic patients, which might influence the satiety and hunger circle (Remely, Aumueller et al. 2014). They evaluated the methylation status of *GPR41* in obese, diabetic and lean individuals and revealed a negative correlation between the body mass and *GPR41* methylation.

1.2.3.2 G Protein-coupled Receptors

Recently, propionate and acetate were reported to be the ligands for two GPRs, GPR41 (free fatty acid receptor 3, FFAR3) and GPR43 (free fatty acid receptor 3, FFAR2), mainly expressed in gut epithelial cells (Brown, Goldsworthy et al. 2003; Le Poul, Loison et al. 2003). Expression of GPCRs in other cell types like adipocytes, immune cells, and sympathetic ganglion has also been reported (Hong, Nishimura et al. 2005; Maslowski, Vieira et al. 2009; Kimura, Inoue et al. 2011). Even though

GPR43 can also be activated by other SCFA, propionate and acetate have been shown to be the most efficient for activating GPR43 (Le Poul, Loison et al. 2003). The fact that *GPR43* is expressed in adipose tissue and intestines suggests that GPR43 may be involved in energy homeostasis. Hong et al. performed a series of studies to elucidate the functions of GPR43 in adipose tissues. They demonstrated that *GPR43* expression was significantly greater in the white adipose tissue of mice on a high-fat diet (41% fat(wt/wt), 36% carbohydrate and 23% protein) compared with normal-fat diet (8.5% fat, 43.7% carbohydrate, and 29.7% protein) fed mice, and suppression of *GPR43* mRNA by RNA interference inhibited adipogenesis, suggesting that SCFA may promote adipogenesis via GPR43 (Hong, Nishimura et al. 2005). Li et al. recently showed that the effect of SCFA in enhancing adipocyte differentiation was not via GPR41 or GPR43 in the stromal vascular fraction of porcine subcutaneous fat (Cox, Yamanishi et al. 2014). Thus, further study is needed to identify molecular pathways of SCFA-stimulated adipogenesis. In brown adipose tissues, which help regulate energy expenditure by thermogenesis, it was reported that *GPR43* KO mice fed a high-fat diet showed improved insulin sensitivity (Bjursell, Admyre et al. 2011). However, others could not detect *GPR43* expression in brown adipose tissues (Kimura, Ozawa et al. 2013). Recently, a series of *in vitro* and *in vivo* studies showed that *GPR43*-deficient mice were obese even when consuming a normal diet, whereas mice overexpressing this receptor specifically in white adipose tissues remained lean, independent of calorie consumption. Importantly, germ-free environment and antibiotic treatment abrogated this effect leading to the hypothesis that the production of SCFA of bacterial fermentation can mediate activation of GPR43, which results in suppression of insulin signaling in the adipose tissue (Kimura, Ozawa et al. 2013).

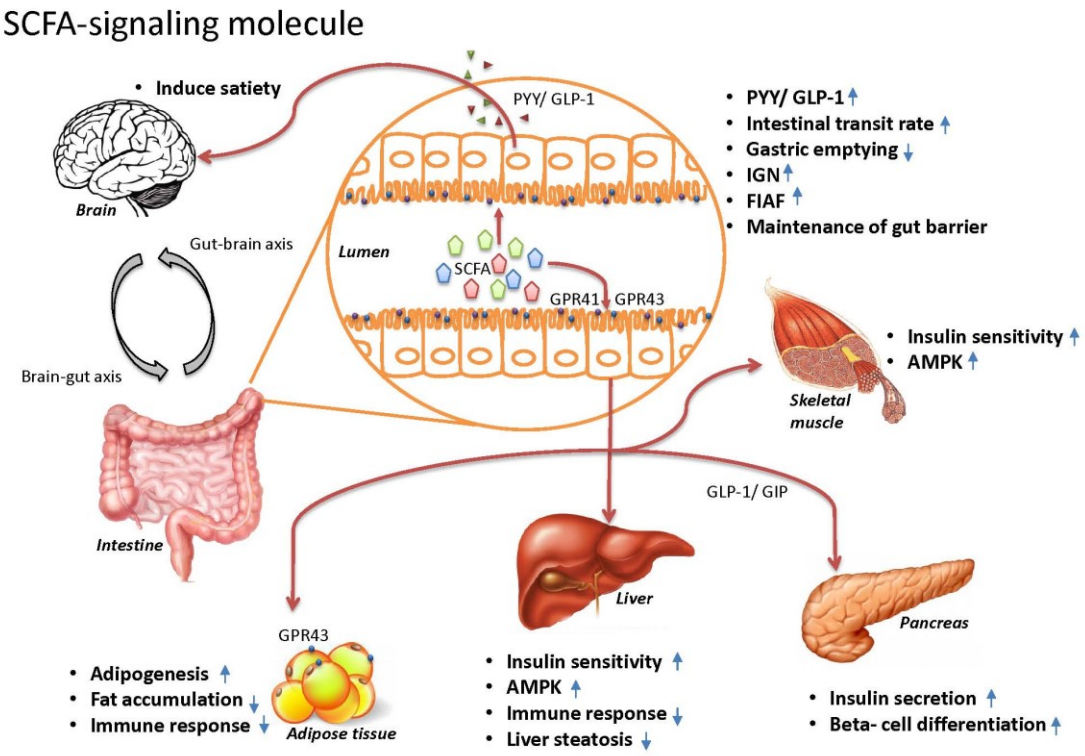

Figure 1.2 Schematic overview of the effects of SCFA on host metabolism as signaling molecules. SCFA improve metabolic related disorders by influencing gene expression as histone deacetylases (HDACs) inhibitors and via G-protein coupled receptors (GPCRs). The three main effects of SCFA are inducing insulin secretion in pancreas, stimulating satiety in brain and suppressing immune responses in adipose tissue and other tissues. In intestine, SCFA improve gut barrier and induce gut hormones (PYY, GLP-1 etc.) production, which will induce satiety in brain and promote insulin secretion in pancreas. SCFA also stimulate FIAF expression in epithelium which will regulate downstream lipid metabolism. In liver and skeletal muscle, SCFA increase insulin sensitivity and increase energy expenditure by activating AMPK. In adipose tissue, FIAF suppresses lipolysis and thus inhibit fat accumulation. SCFA also reduce the release of proinflammatory cytokines and weaken immune cells infiltration in adipose tissue. Therefore, SCFA regulate food intake, energy expenditure and reduce inflammation to promote host homeostasis.

1.2.4 SCFA, Host Metabolism and Metabolic Diseases

Short chain fatty acids, among which butyrate is the most well studied, modulate different processes, including cell proliferation and differentiation (Zaibi, Stocker et al.

2010), gut hormone secretion and immune responses (Atarashi, Nishimura et al. 2008; Maslowski, Vieira et al. 2009), and are thus involved in the regulation of host homeostasis. Although metabolic disorders are caused by multiple factors, gut microbiota dysbiosis and improper production of SCFA may play a vital part in the pathogenesis of metabolic disorders. Overall, treatments that enhance microbial production of SCFA or direct supplementation show improvement in metabolic outcomes (Yamashita, Fujisawa et al. 2007; Gao, Yin et al. 2009; Lin, Frassetto et al. 2012).

1.2.4.1 SCFA and Satiety

High intake of dietary fiber has been indicated to prevent the development of obesity and help reduce body weight (Liu, Willett et al. 2003; Grube, Chong et al. 2013). One of the mechanisms by which dietary fiber helps reduce obesity risk is via SCFA-mediated modulation of satiety hormones including glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) that regulate food intake and energy harvest (Tolhurst, Heffron et al. 2012). As an incretin produced by intestinal L cells, GLP-1 increases insulin secretion and decreases food intake by inducing satiety in the brain (Barrera, Jones et al. 2011). PYY is mainly produced by L cells in the distal gut and known for its role in regulating pancreatic and gastric secretion, gut motility, insulin secretion and control of appetite in central nervous system (Manning and Batterham 2014). Lin et al. observed that oral administration of butyrate and propionate in mice significantly increased the plasma GLP-1 and gastric inhibitory polypeptide (GIP), accompanied by a modest increase in PYY, leading to improved insulin sensitivity (Lin, Frassetto et al. 2012). Microbial transplantation from lean donors increases insulin sensitivity in individuals with metabolic syndrome along with the elevated levels of butyrate-producing microbes (Vrieze, Van Nood et al. 2012). The underlying mechanisms have yet to be fully established and the possible mechanisms will be discussed below.

In the intestine, it is indicated that the effects of SCFA on GLP-1 and PYY production are via GPR43 (Karaki, Mitsui et al. 2006; Nohr, Pedersen et al. 2013). *GPR43* and *GPR41* were abundantly expressed in GLP-1 and PYY secreting L-cells. Whether GPR41 and GPR43 reside on the apical or basolateral membrane of L-cells or whether they primarily detect luminal or plasma SCFA are to be tested (Tolhurst, Heffron et al. 2012). *GPR43*- and *GPR41*-knockout mice exhibited reduced SCFA-mediated GLP-1 secretion accompanied with reduced insulin secretion and impaired glucose tolerance consistent with their observation of lower GLP-1 secretion *in vitro* (Tolhurst, Heffron et al. 2012). *GPR41* or *GPR43* are expressed in many types of cells including adipocytes, immune cells and pancreatic β and α-cells, therefore global knockout of these receptors may affect glucose metabolism, inflammation, or pancreatic β-cell functions, which would themselves impact on glucose tolerance. In *GPR41*-deficient mice, expression of PYY is reduced, resulting in increased gut motility and reduced energy extraction from SCFA (Inoue et al., 2014). Conventionalization of germ-free mice with two predominant bacteria derived from the human gut significantly increased PYY. This effect was suppressed in germ-free *GPR41* KO mice when colonized with the two bacteria even though the diet consumption was similar in both groups, indicating that SCFA activation of GPR41 is required for inducing PYY secretion (Samuel, Shaito et al. 2008). Furthermore, the body weight and fat pad weight of *GPR41* KO mice were significantly reduced compared with wild-type littermates, and, this effect was abolished in a germ-free environment; consistent with the *GPR43* KO mice raised in germ-free conditions, indicating that SCFA derived from metabolism of gut microbiota exert receptor-mediated effects on host adiposity (Samuel, Shaito et al. 2008). To the contrary, another group found that absence of GPR41 increases body fat content in male mice when fed a high-fat diet without a difference in food intake. Gut-derived SCFA actually raised energy expenditure and helped to protect against obesity by activating GPR41 (Bellahcene, O'Dowd et al. 2013). In adipose tissue, it was shown

that propionate-stimulated activation of GPR41 increases the release of leptin, a hormone controlling the sensation of hunger as well as energy expenditure. In addition, the secretion of leptin was increased through over-expression of exogenous *GPR41* and decreased by siRNA-mediated knockdown of *GPR41* (Xiong, Miyamoto et al. 2004). However, two other groups could not detect the *GPR41* expression in adipose tissue (Hong, Nishimura et al. 2005; Kimura, Ozawa et al. 2013), suggesting that the interaction between GPR41 and leptin may be indirect.

1.2.4.2 SCFA and Inflammation

Obesity and diabetes are characterized by low-grade inflammation with increased peripheral blood concentrations of cytokines, such as interleukin (IL)-1, IL-6, or tumor necrosis factor-alpha (TNF-α). These inflammatory molecules are up-regulated in insulin-target tissues, including liver, adipose tissue, and muscles, thus contributing to insulin resistance (reviewed by (Puddu, Sanguineti et al. 2014)). SCFA, particularly butyrate, were shown to have anti-inflammatory effects (Kendrick, O'Boyle et al. 2010; Canani, Costanzo et al. 2011) through GPCRS. The expression of *GPR43* has been observed in neutrophils, eosinophils and activated macrophages. *GPR43* deficient mice had a profoundly altered inflammatory response, which included exacerbated inflammation in a model of allergic airway inflammation as well as in colitis models (Maslowski, Vieira et al. 2009). In addition, Sina et al. showed the importance of GPR43 on leukocyte migration and cytokine secretion in an inflammatory model (Sina, Foerster et al. 2009). SCFA also direct the development of extrathymic anti-inflammatory regulatory T cells (Arpaia, Campbell et al. 2013). As the only known ligands for GPR43, SCFA also control the generation of colonic regulatory T cells and protect against colitis in a GPR43-dependent manner (Smith, Howitt et al. 2013). SCFA are also beneficial in other ways. SCFA, particularly butyrate, inhibit activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) as HDAC inhibitors.

Germ-free mice with very low concentration of SCFA showed exacerbated or poorly resolving responses in many inflammatory models (Maslowski, Vieira et al. 2009; Chervonsky 2010), similar to the responses of the *GPR43* KO mice. SCFA were also shown to have anti-inflammatory effects by reducing chemotaxis and cell adhesion and thus SCFA at least partially prevent infiltration of immune cells into adipose tissue(Meijer, de Vos et al. 2010; Kim, Kim et al. 2014). Treatment with propionic acid in overweight subjects reduced proinflammatory cytokine and chemokine secretion from human adipose tissue as well as from macrophages. However, the effects of propionate were dependent on GPR41 but not GPR43 (Al-Lahham, Roelofsen et al. 2012).

1.2.4.3 SCFA Regulate Glucose Metabolism

Plasma glucose levels are determined by food intake, gluconeogenesis, and uptake by multiple organs. As discussed above, in addition to the fact that propionate can be used for gluconeogenesis in liver, the major effects of SCFA on glucose metabolism are through the GPCR by influencing the gut hormones PYY and GLP-1. Intestinal gluconeogenesis (IGN) has also been identified to have beneficial effects on glucose and energy homeostasis including improved glucose tolerance (De Vadder, Kovatcheva-Datchary et al. 2014). SCFA, especially propionate and butyrate, may exert some of their effects by directly modulating IGN. It was reported that butyrate activated IGN gene expression through a cAMP-dependent mechanism, while propionate, itself a substrate of IGN, activates IGN gene expression via a gut-brain neural circuit involving *GPR41*. The benefits on glucose and energy control induced by SCFA or dietary fiber in normal mice were absent in mice deficient for IGN (intestinal specific deletion of G6Pase catalytic subunit), despite similar modifications in gut microbiota composition. Therefore, regulation of IGN seems to be the key to beneficial metabolic effects associated with SCFA and soluble fiber (De Vadder, Kovatcheva-Datchary et al. 2014).

1.2.4.4 SCFA Regulate Fatty Acid Metabolism

Adipocytes are key target cells concerning the prevention and treatment of Type 2 diabetes. Impaired regulation of lipolysis and accumulation of lipid intermediates may contribute to obesity-related insulin resistance and Type 2 diabetes. In adipose tissue, the hydrolysis of triacylglycerol into free fatty acids and glycerol, known as lipolysis, often leads to excessive release of fatty acids to the plasma and decreased insulin sensitivity in overweight or obese individuals (Nielsen, Guo et al. 2004; Jocken, Goossens et al. 2013). Heimann et al. recently showed that SCFA inhibit lipolysis and the basal and insulin-stimulated lipogenesis as well as enhancing the insulin-stimulated glucose uptake in primary adipocytes that might be beneficial for prevention of dysfunctional adipocytes associated with insulin resistance as seen in obese and diabetic individuals (Heimann, Nyman et al. 2014).

The series of experiments conducted by Bäckhed and his colleagues showed that adiposity increased in the conventionalized germ-free mice and was caused by the microbial suppression of *Fiaf* (Fasting-induced adipocyte factor or ANGPTL4), an inhibitor of lipoprotein lipase (LPL). LPL promotes release of fatty acids from circulating chylomicrons and very low-density lipoproteins (VLDL), which results in their storage as triglycerides in the adipose tissue. FIAF inhibition of LPL therefore reduces fat storage. One reason why germ-mice mice are protected from obesity is the elevated expression of *Fiaf* in the intestine (Backhed, Ding et al. 2004; Backhed, Manchester et al. 2007). A recent study showed that transcription and secretion of FIAF in human colon adenocarcinoma cells is induced by high concentration of SCFA via nuclear receptor peroxisome proliferator activated receptor γ (PPARγ) and butyrate is the strongest activator followed by propionate and acetate(Alex, Lange et al. 2013), which provides another mechanism through which SCFA prevent adiposity. SCFA have been shown to increase the AMPK activity in liver and muscle tissue,

which will activate peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α expression that is important in regulation of cholesterol, lipid, and glucose metabolism (Yamashita, Fujisawa et al. 2007; Gao, Yin et al. 2009). SCFA may also influence fatty acid metabolism via GPR43 as adipocytes treated with acetate and propionate exhibited a reduction in lipolytic activity that is not observed in *GPR43* deficient mice (Hong, Nishimura et al. 2005; Gao, Yin et al. 2009). Li et al. recently showed that the effect of SCFA in enhancing adipocyte differentiation was not via GPR41 or GPR43 in stromal vascular fraction of porcine subcutaneous fat (Cox, Yamanishi et al. 2014). Thus, further study is needed to identify molecular pathways of SCFA-stimulated adipogenesis.

In summary, SCFA are important microbial products and affect a range of host processes-including energy harvest, host-microbe signaling and gut integrity. Since SCFA have the effect of inhibiting food intake, improving insulin sensitivity and increasing energy expenditure in the host, they are regarded as healthy microbial products that prevent the development of obesity and diabetes. Even though gastric bypass surgery is still regarded as the most effective treatment of obesity, SCFA supplementation or probiotic intake that promote SCFA production can also be considered as safe treatment alternatives to prevent and treat obesity (Yadav, Lee et al. 2013).

1.2.5 Microbe-induced Metabolic Inflammation

While acute inflammation is essential to heal the body after an injury or infection, extensive research over the past decade has made it clear that chronic, low-grade inflammation contributes to the onset of metabolic diseases including obesity, diabetes, cardiovascular diseases and non-alcoholic steatohepatitis (NASH) (Hotamisligil 2006; Szabo, Bala et al. 2010; Lumeng and Saltiel 2011; Tarantino 2014). Plasma bacterial endotoxin (e.g., LPS) originating from the intestinal

microbiota has been implicated in inducing metabolic inflammation resulting in insulin resistance in obesity and type 2 diabetes (Basu, Haghiac et al. 2011; Lassenius, Pietilainen et al. 2011). The role of LPS in inducing inflammation and metabolic disease is depicted.

LPS is a major component of the outer membrane of Gram-negative bacteria and is released upon microbial death (Rietschel, Brade et al. 1996). The lipid A domain, which is highly conserved, is the ligand that activates Toll-like receptor 4 (TLR4) that is present on the cell membrane of immune cells, adipocytes and epithelial cells (Frantz, Kobzik et al. 1999; Devaraj, Dasu et al. 2008; Vitseva, Tanriverdi et al. 2008; Ioannidis, Ye et al. 2013). Recognition of TLR4 activates downstream proinflammatory responses (Dixon and Darveau 2005) essential for the host to defend against bacterial infection. The concentration of LPS is low in the circulation of healthy individuals; however, it has been shown that obese subjects and diet-induced animal models have increased circulating LPS levels (Cani, Amar et al. 2007; Erridge, Attina et al. 2007; Pussinen, Havulinna et al. 2011). Importantly, increased adiposity and insulin resistance usually seen in animals fed a high fat diet can also be observed in mice chronically infused with LPS; indicating that LPS triggers adiposity and diabetes via activation of the immune system (Cani, Amar et al. 2007).

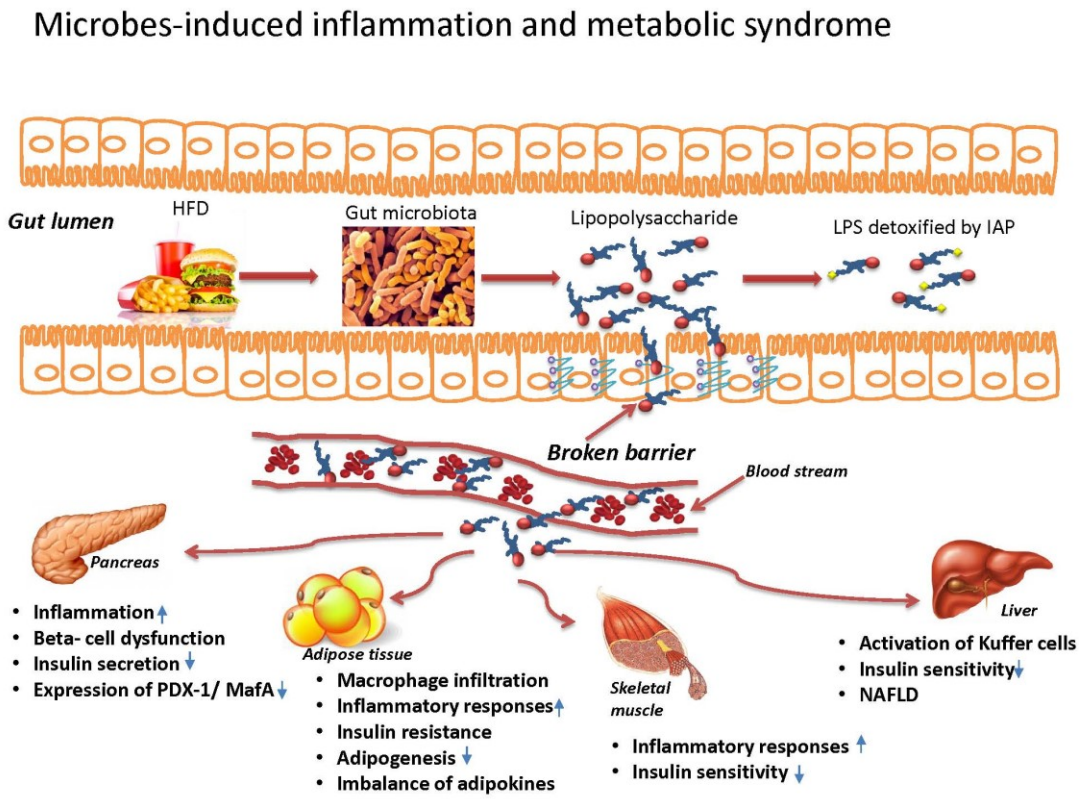

Figure 1.3 The role of LPS in inducing inflammation and metabolic diseases. High fat intake increases plasma lipopolysaccharides (LPS). LPS translocates through gut epithelium with chylomicrons and induces local inflammation, which impairs tight-junctions increasing gut permeability. Once circulating in the blood, LPS activates immune cells and induces proinflammatory cytokine production. When plasma LPS reaches peripheral organs, LPS activates TLR4 on the cell membranes and leads to downstream inflammatory responses and immune cell infiltration which impairs insulin signaling and insulin related gene expression in pancreas, adipose tissue, liver and skeletal muscle.

1.2.6 Consequences of Inflammation on Metabolism

Exposure to LPS results in innate immune activation in many cell types, especially dendritic cells and macrophages. The production and secretion of many pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin 1 (IL-1) and IL-6 have been indicated to play a role in the pathogenesis of chronic inflammation in obesity, insulin resistance and diabetes (Musso, Gambino et al. 2011) through their effects on various tissues. Chronic inflammation causes damage to pancreatic beta cells (Hohmeier, Tran et al. 2003; Eguchi, Manabe et al. 2012),

hepatocytes (Gieling, Wallace et al. 2009), adipocytes (Hotamisligil 2006) and vascular endothelial cells (Hansson 2005).

Insulin signaling is one of the first processes affected by LPS arriving in the circulation. Insulin signaling is a complex process that involves multiple pathways and cascades of phosphorylation events. Interference with these signaling pathways can alter insulin production or sensitivity and lead to the development of insulin resistance (Hotamisligil 2006). TLR4 is expressed in insulin target tissues, including skeletal muscle, adipose tissue and liver. Activation of TLR4 by LPS activates proinflammatory kinases such as c-Jun N-terminal kinase (JNK), IκB kinase (IKK), and p38 mitogen-activated protein kinase (MAPK) that target the insulin receptor substrate (IRS) for serine phosphorylation, which in turn impairs insulin signal transduction. The inflammatory kinases also regulate downstream transcription factors, such as NF-κB and interferon regulatory factor (IRF), resulting in increased production of cytokines. The increased cytokines and chemokines induced by TLR4 promote further insulin-desensitization within the target cells and in other cells via paracrine and systemic effects (Reviewed by(Kim, Kim et al. 2014).

The pancreas is at the heart of glucose metabolism, given its role as the sole source of insulin and glucagon. Insulin is secreted in response to nutrients being absorbed into the bloodstream after a meal, particularly glucose. Insulin then instructs cells of the body to take up glucose and as well activates anabolic pathways. The pancreas can suffer directly from inflammation, as well as indirectly as a result of insulin resistance in other tissues. Recent studies have shown that macrophage infiltration in pancreas is triggered by TLRs 2 and 4, which leads to greater expression of inflammatory cytokines, IL-1β, TNF-α and interferon-γ (IFN-γ) (Ehses, Boni-Schnetzler et al. 2008; Donath, Boni-Schnetzler et al. 2010; Cucak, Mayer et al. 2014; Westwell-Roper, Nackiewicz et al. 2014). Critical events in the progression to diabetes were also observed in chronic inflammation models, including reduced insulin secretion and β

cell apoptosis and decreased islet mass, which are due to a pathological activation of the innate immune system by metabolic stress (Donath, Boni-Schnetzler et al. 2010; Wu, Wu et al. 2013). These all suggest that chronic inflammation triggers insulin resistance and impaired islet function. As a low-grade inflammation activator, LPS has shown effects on β cell dysfunction in a rat model infused with LPS (Hsieh, Chan et al. 2008). Recently, Amyot et al. demonstrated that LPS could inhibit β cell transcriptional activity in a TLR4-dependent manner and via NF-κB signaling (Amyot, Semache et al. 2012).

The reduced capacity of cells to take up glucose in response to insulin results in elevated blood glucose levels, which in-turn activates further insulin secretion causing stress on pancreas. Skeletal muscle consumes the largest amount of glucose in the body. Probiotic strains of bacteria have been administered to mice and showed indications of greater insulin sensitivity including increased expression of glucose transporter type 4 (GLUT4) and glycogen synthesis related enzymes (Kim, Kim et al. 2014).

Among metabolic tissues affected by inflammation, adipose was the first described and most studied in metabolic disorders. Adipose tissue acts not only as a storage depot for energy, but also secretes hormones, cytokines, and chemokines that promote inflammation, and conversely adipokines and adiponectin that promote insulin sensitivity (Hotamisligil 2006). Generally, LPS influences the adipose metabolism in four ways. First is the recruitment of macrophages to the adipose tissue, resulting in amplified inflammation (Locati, Mantovani et al. 2013). Second is the suppression of insulin signaling in adipose tissue, as discussed above, as well as through activation of phosphoinositide 3-kinase (PI3K)/Akt (Wakayama, Haque et al. 2014). Third, LPS has been shown to induce lipolysis in human adipocytes via both IKKβ/ NF-κB pathway and protein kinase A (PKA)/hormone-sensitive lipase (HSL) pathways (Grisouard, Bouillet et al. 2012). Finally, LPS regulates adipocyte differentiation by

downregulating the activity of PPARγ, which is essential to adipogenesis and to maintenance of adipocyte gene expression and function (Luche, Cousin et al. 2013)

The liver plays a key role in distributing nutrients to the rest of the body. This includes the storage and release of glucose and packaging of fatty acids in the form of VLDL and LDL. There are several liver diseases where microbe-induced inflammation plays an important role including NASH, NAFLD. Bacterial translocation has been associated with fat accumulation in the liver and liver fibrosis. Removal of Kuppfer cells, and thus the primary mechanism to induce inflammation in the liver, prevents the development of hepatic steatosis and insulin resistance in response to HFD. The production of TNF-α by Kuppfer cells induced hepatocyte triglyceride accumulation, fatty acid esterification and reduced fatty acid oxidation and insulin responsiveness (Huang, Metlakunta et al. 2010). Furthermore, TLR4 deficient mice showed reduced liver injury in a model of NASH (Rivera, Adegboyega et al. 2007). Inflammation also impacts the ability of macrophages to facilitate cholesterol removal through reverse cholesterol transport (Majdalawieh and Ro 2009). Bacterial overgrowth is one reason for increased bacterial translocation, and thus activation of Kuppfer cells in the liver leading to liver disease (Pardo, Bartoli et al. 2000).

1.2.7 LPS, High Fat Diet and Gut Permeability

HFD has been shown to cause metabolic endotoxemia in animals and humans (Cani, Amar et al. 2007; Ghanim, Abuaysheh et al. 2009; Pendyala, Walker et al. 2012), but the underlying molecular mechanisms remain incompletely understood. In healthy humans, postprandial plasma LPS concentration increased significantly compared with the fasting level after a high-fat meal or a mixed meal containing emulsified fat (Erridge, Attina et al. 2007; Laugerette, Vors et al. 2011). In order to establish whether plasma LPS levels are based on energy or fat intake, fasting normal subjects were given drinks containing glucose, 100% saturated fat or orange juice. Elevated LPS

levels were only associated with the fat intake (Deopurkar, Ghanim et al. 2010). LPS reaches the blood from the intestinal lumen through paracellular and transcellular routes. Impaired epithelial integrity permits increased paracellular transport of LPS. Transcellular transport occurs through receptor-mediated endocytosis (Mani, Weber et al. 2012). Ghoshal et al. demonstrated that intestinal epithelial cells internalize LPS from the apical surface, which is then transported to the Golgi apparatus where it complexes with chylomicrons. The chylomicron–LPS complex is then secreted into mesenteric lymph and makes its way into the systemic circulation (Ghoshal, Witta et al. 2009). In the bloodstream, LPS binds to many macromolecules such as albumin, low-density lipoprotein (LDL), high-density lipoprotein (HDL) and LPS-binding protein. Once combines with LPS-binding protein (LBP), LPS can be transferred to membrane-bound or soluble CD14, thereby enabling interactions with TLR4 on cell membranes and downstream inflammatory responses (Beumer, Wulferink et al. 2003).

The intestinal epithelium primarily serves as a dynamic barrier, which in the course of its normal function maintains regulated absorption of nutrients and water while excluding potential pathogens. The gut integrity is maintained by commensal bacteria, epithelial intestinal cell factors and gut hormones (GLP-1, PYY etc.) which could be disrupted when exposed to HFD (Reviewed by (Bleau, Karelis et al. 2014). Although evidence to support increased gut permeability in human subjects is minimal, many animal studies have shown increased LPS levels in the lamina propria, mesenteric adipose tissue and blood resulting from gut barrier dysfunction and bacterial translocation in genetic obese or diet-induced models, reflected by reduced expression of tight junction proteins zona occuldens (ZO)-1, occludin, claudin-1, claudin-3 and junctional adhesion molecule 1 (JAM-1) (Brun, Castagliuolo et al. 2007; Teixeira, Collado et al. 2012; Bleau, Karelis et al. 2014). In NASH, increased intestinal permeability, increased LPS levels and tight-junction alterations are also observed (Miele, Valenza et al. 2009; Ilan 2012). Indirect evidence that shows the association between gut permeability and LPS translocation was from probiotic research.

Akkermansia muciniphila has been shown to restore gut barrier function, and in doing so reduced serum LPS decreasing the effects of diet-induced obesity. Without a change in food intake mice showed improved insulin sensitivity, decreased body weight gain, fat mass development, and fasting hyperglycemia (Everard, Belzer et al. 2013).

1.2.8 LPS Detoxification

To prevent inflammation induced by LPS the body has developed several strategies to detoxify it. The apical brush-border enzyme intestinal alkaline phosphatase (IAP) detoxifies a variety of bacterial components, including LPS, CpG DNA, and flagellin through dephosphorylation (Chen, Malo et al. 2010). IAP is produced exclusively in villus-associated enterocytes and secreted into the gut lumen (Alpers, Mahmood et al. 1994). Several *in vitro* and *in vivo* studies have shown that IAP is essential in LPS detoxification and preventing bacterial invasion across the gut mucosal barrier. Exogenous IAP not only significantly inhibited LPS-induced inflammatory cytokine production, but also prevented and reversed metabolic syndrome in mice (Goldberg, Austen et al. 2008; Kaliannan, Hamarneh et al. 2013; Lee, Chun et al. 2014). Differences in IAP activities and inflammation between diet-induced obesity prone and resistant rats were also documented (de La Serre, Ellis et al. 2010). In addition, IAP deficient mice showed increased gut permeability caused by enhanced LPS translocation (Goldberg, Austen et al. 2008; Kaliannan, Hamarneh et al. 2013) and gained more weight when fed with a HFD (Narisawa, Huang et al. 2003). Together these studies demonstrate IAP dephosphorylates LPS preventing inflammation, which would otherwise lead to further damage in other organs that are related to the pathogenesis of metabolic diseases. Interestingly, as a defense protein it has no effect on live bacteria (Chen, Malo et al. 2010).

Once in the systemic circulation, LPS can be deactivated or detoxified by immune

cells, such as macrophages, or Kupffer cells present in the liver or splenic cells or by binding with acute phase proteins (Satoh, Ando et al. 2008; Buttenschoen, Radermacher et al. 2010). Bactericidal/permeability increasing protein (BPI), which is widely expressed in epithelial cells, is capable of binding and neutralizing LPS (Canny, Levy et al. 2002). The high affinity of BPI for the lipid A moiety of LPS targets its cytotoxic activity to Gram-negative bacteria by preventing the interaction of lipidA with other pro-inflammatory molecules (Weiss 2003).

1.3 Early Life Programming of Metabolic Disease

1.3.1 Early Life Environmental Stimuli and Beta Cell Function

Epidemiological studies in humans have shown that an adverse maternal environment during embryonic development is associated with an increased risk of obesity, type 2 diabetes (T2D), cardiovascular disease and metabolic syndrome (Wang et al., 2013). In addition, it has been shown that perturbation in early postnatal period can also exert long-term consequences for the development of metabolic disease in later life (Lucas et al., 1998; Soubry et al., 2013). Barker et al and Hales et al observed that low birth weight was associated with high prevalence of T2D and cardiovascular diseases. These findings let them turn to the concept of programming in early life and came up with the 'thrifty phenotype hypothesis', which postulates that poor fetal and infant nutrition are the insult that drives the metabolic changes, including decreased β-cell mass, restricted fetal growth and impaired adult β-cell function (Barker et al., 1989; Hales et al. 1991; Hales et al., 2001). In the last two decades, the hypothesis has been confirmed by many epidemiological studies from humans and animal models, showing that maternal over-nutrition and under-nutrition is linked to abnormal metabolism and body compositions in offspring (Cottrell et al., 2008; Barouki et al., 2012). Children born to diabetic mothers are also more likely to become diabetic compared with internal controls (offspring born before the mothers became diabetic

or with low risk of diabetes) (Dabelea et al., 2000).

Under normal conditions, pancreatic islet β-cells are triggered to release insulin to the blood to regulate glucose homeostasis after a meal. Thus, β-cells have a key role in modulating glucose concentration by insulin secretion. Beta cell failure marks the irreversible deterioration of glucose regulation and ultimately results in T2D (Kahn et al., 2003). Various animal studies have demonstrated that a maternal diet deficient in proteins during pregnancy and lactation alters normal development of the pancreas and insulin sensitivity, which leads to glucose intolerance and insulin resistance in offspring, accompanied with reduced size of islets of Langerhans and β-cell mass. Deficient maternal diet also alters the duration of the cell cycle of pancreatic β-cell cells and decreases the expression of genes related to insulin production, including glucose transporter 2 (*GLUT2*) and transcription factors like pancreatic and duodenal homeobox-1 (*PDX-1*) (reviewed by Sosa-Larios et al., 2015). It becomes clear that the developmental environment induces disease state metabolic phenotypes via a combination of genetic, physiological and epigenetic mechanisms (Wang et al., 2013).

1.3.2 Epigenetics in the Pathogenesis of Metabolic Syndrome

Generally, events in early life may programme metabolic pathways in three ways. One mechanism is that the early environment could have a permanent effect on tissue function through alterations in organ structure resulting from a stimulus or insult during a critical window. An example is the structural changes of endocrine organs and the restricted β-cell mass has been observed in several intrauterine growth restriction (IUGR) models (Tarry-Adkins et al., 2011). Another mechanism is the through epigenetic modifications of gene expression that is set by an early stimulus or insult with long-term consequences for function. In addition, permanent effects on the regulation of cellular aging, such as increases in oxidative stress also contribute to long-term alterations of development (Lucas et al., 1998; Tarry-Adkins et al., 2011).

As mentioned above, there is a critical window in the early life for the development of endocrine organs, immune system, intestinal structure and gut microbiota establishment. It is known that when the fetus is exposed to an adverse condition *in utero*, it adapts to the environment to maintain homeostasis and assure survival (Gluckman et al., 2008). Individual developmental adaption to the environment, also called developmental plasticity, can increase the risk of disease in later stages of life (West-Eberhard et al., 2003). Developmental plasticity requires stable modulation of gene expression and this appears to be mediated by epigenetic processes (Gluckman et al., 2011). Therefore, over the last decade, there has been increasing interest in studying the effects of epigenetics in the development of complex conditions. Unlike genetic modifications that change the base sequence of DNA, epigenetic modifications refer to chemical modifications to DNA methylation, histone or chromatin modifications and regulation by microRNAs without altering nucleotide sequence. DNA methylation occurs at 5-methyl cytosine in the context of CpG dinucleotides, also called CpG sites. It is the best studied epigenetic modification and governs transcriptional regulation and silencing (Suzuki et al., 2008). Epigenetics affect how cells interpret genes by switching genes on and off, which are typically caused by external or environmental factors (Allis et al., 2007). Epigenetic changes are reversible and can be reprogrammed in response to environmental stimuli. In general, these changes are preserved during somatic cell division and most changes only occur within the course of one individual lifetime. Some of the epigenetic modifications either alone or in combination may be responsible to environmental programming (Gilbert et al., 2012).

With the increasing availability of affordable tools for genome-wide association studies (GWAS), there has been a rapid expansion of epigenetic analysis in humans, mainly focused on the identification of site-specific DNA methylation differences that are associated with metabolic phenotypes. A GWAS profiling pancreatic islets from

T2D identified 276 differentially methylated CpG sites that are associated with T2D (Volkmar et al., 2012). In another genome-wide DNA methylation analysis, 1649 CpG sites and 853 genes were identified as differentially methylated in pancreatic islets from T2D and non-diabetic donors (Dayeh et al., 2014). In the wide range of these studies, epigenetic effects have been examined at various times including embryonic and postnatal development period and different sample types including blood, pancreas, muscle, colon and adipose tissue (van Dijk et al., 2015). The major limitation in many human studies is that epigenetic marks are often assessed in peripheral blood, rather than the metabolically relevant tissues, such as pancreas and adipose tissue. It is known that DNA methylation changes associated with obesity or T2D vary depending on the tissue studied. Different cell populations have distinct epigenetic signatures (Heilbronn et al., 2012). An example is that the methylation of hypoxia inducible factor 3 gene (*HIF3A*) related to BMI in the adipose tissue was more pronounced than in blood (Dick et al., 2014). Therefore, different animal models for maternal nutrition studies and other early environmental stimuli that mimic human conditions are developed and have provided evidence of metabolic and epigenetic changes in offspring (van Dijk et al., 2015).

1.3.3 Epigenetic Modifications of Genes Related to Metabolic Syndrome

With intense human and animal model studies in recent years, a number of genes in the early life are identified to be particularly important for the development of metabolic disease. One of the most significant studies was conducted in children born to mothers who underwent under-nutrition during pregnancy in the World War II. These individuals have shown to be at higher risk of obesity and glucose intolerance, associated with a reduced methylation of the imprinted gene insulin-like growth factor 2 (*IGF2*) in these individuals as adults, depending on the timing of the exposure to famine in early life (reviewed by van Dijk et al., 2015). IGF-2 shares similar structure to insulin with the function of promoting growth during gestation and assisting

development of fetal pancreatic β-cells through the Irs1/Irs2-PI3K-Akt pathway (Fujimoto et al., 2009). It is imprinted through the differentially methylated region (DMR) and only paternal allele is expressed (Huang et al., 2012). In T2D, pancreatic islets of Langerhans are of central importance in the developmental process. It has been reported that overexpression of *IGF2* can induce β-cell dysfunction and transgenic mice overexpressing *IGF2* specifically in β-cell developed a pre-diabetic state (Casellas et al., 2015). Epigenetic association between methylation at the *IGF2/H19* imprinting region at birth in blood cells and pancreatic islets with growth characteristics and obesity in later life has been consistently observed (Drake et al., 2012; Huang et al., 2012; Ding et al., 2012; Soubry et al., 2013). A contradictory finding in mice suggests that there is no effect of maternal nutrition in *IGF2* DMRs (Sferruzzi-Perri et al., 2013). Despite the interest in studying the association between *IGF-2* imprinting and metabolic disease in recent years, the role of *IGF2* in the pathogenesis is not fully understood.

In addition to *IGF-2*, one of the best known examples of epigenetic changes leading to diabetes in the offspring born to nutrition restricted mothers is the silencing of *PDX-1* in β-cells (Park et al., 2008). *PDX-1* is a transcription factor that is essential for pancreatic development, β-cell differentiation, insulin production and glucose homeostasis by regulating expression of other genes, such as insulin, *GLUT2* and glucokinase (Murtaugh et al., 2007). *PDX-1* expression in pancreatic islets can reprogram cells toward the β-cell fate (Yang et al., 2011). Beta-cell *PDX-1* specific inactivation has been reported to cause β-cell dysfunction and impair glucose homeostasis in rodents (Brissova et al., 2005; Arantes et al., 2002). In an IUGR rat model, expression of *PDX-1* is downregulated by 50% after birth into adulthood, leading to the progressive reduction in β-cell mass, defects in insulin secretion and T2D development (Stoffers et al., 2003). Consistently, human studies also linked the development of T2D to the reduction of *PDX-1* expression, highlighting the crucial role that this developmental factor plays in adulthood (Holland et al., 2005). In

humans, increased methylation of *PDX-1* and decreased expression of *PDX-1* were observed in pancreatic islets from T2D patients compared with controls (Yang et al., 2012). A genome-wide DNA methylation analysis also revealed increased methylation of *PDX-1* and *INS* (the gene that encodes for insulin) in T2D patients (Dayeh et al., 2014). In an IUGR rodent model, epigenetic changes were found to be responsible for *PDX-1* silencing in pancreatic islets. It was addressed that during the neonatal period, epigenetic changes and the reduction of *PDX-1* can be reversed by the inhibition of HDAC (Park et al., 2008).

1.3.4 Antibiotics and Early Life Programming

The exposure to antibiotics in different situations has led to both reductions and increases in metabolic issues. In many cases where increased intestinal permeability and reduced barrier function have been implicated in metabolic disease, antibiotic treatment is found to be protective against glucose intolerance and insulin resistance (Zhao et al., 2013). However, there are also studies that have shown disturbances in the microbiota induced by antibiotics can contribute to development of obesity (Jess et al., 2014). A recent study showed that low levels of antibiotics during a critical window after birth have a programming effect that results in adverse long-term metabolic outcomes (Cox, Yamanishi et al. 2014). It is yet unclear how this early life antibiotic treatment increases adiposity later in life as changes were seen in both immunity and hepatic gene expression relevant to adiposity. It was noted, however, that the microbiota after antibiotic withdrawal was similar to controls that had never received antibiotics whereas adiposity phenotypes were maintained, suggesting that the effect was not associated with persistent changes in the microbiota (Cox, Yamanishi et al. 2014).

Compared with much progress made in maternal nutrition and long-term metabolic consequences, little is known about the mechanisms of antibiotic-induced dysbiosis

and the programming of metabolic syndrome. How epigenetic modifications are involved in early life antibiotic-induced microbial programming of metabolic syndrome pathogenesis later in life is still under investigation. In human studies, antibiotic exposure during pregnancy was shown to be associated with low birth weight, which usually increases the risk of adult-onset chronic diseases, including obesity, cardiovascular disease, T2D and some cancers. Notably, methylation of DMRs of *IGF2* and some other imprinted genes were associated with maternal antibiotic use, which may predispose to metabolic syndrome later in life (Vidal et al., 2013). A whole-genome methylation study recently revealed a methylation pattern associated with microbial predominance. The genes with differentially methylated promoters in which *Firmicutes* was dominant were linked to risk of diseases, including obesity, cardiovascular diseases, lipid metabolism and inflammatory responses (reviewed by Kumar et al., 2014). Recent reports suggest that during the critical and sensitive pre- and postnatal period, microbiota may influence genomic reprogramming via its metabolites. *Faecalibacterium prausnitzii* and *Eubacterium rectale*/*Roseburia* spp., which belong to the *Firmicutes*, are major contributors of butyrate, which regulates gene expression by histone modifications (Canani et al., 2012). Another important microbial metabolite LPS is also suggested to have a significant role in the epigenetic regulation of immune and intestinal cells (Angrisano et al., 2010). A recent study that focused on the microbial epigenetic regulation of immune system revealed lower methylation of TLR4 in obese individuals and lower methylation of TLR2 in T2D patients. In this study, a higher ratio of *Firmicutes/Bacteroidetes* and less abundance of *Faecalibaterium prausnitzii,* known for butyrate synthesis and insulin sensitivity improvement abilities, were observed in T2D patients (Remely et al., 2014). The methylation levels of both TLRs were significantly correlated with body mass, suggesting that changes in intestinal microbiota are involved in the epigenetic regulation of inflammatory responses, which is associated with the development of metabolic disease (Remely et al., 2014). Another evidence from this group showed that the differential microbiota in obese and

type 2 diabetes were associated with a lower methylation of *GPR41*, the receptor for SCFA, suggesting that microbiota may regulate host metabolic changes via epigenetic modification by its products (Remely et al., 2014).

1.4 Research Objectives

In the past few years, antibiotic exposure in the early life, especially the first 6 months, has been linked with metabolic syndrome development in several epidemiological studies in Europe and U.S. With the high prescription rates of antibiotics to treat infections, little is known about how antibiotic exposure alters host metabolism and increases the risk for metabolic disease. If the cellular and molecular mechanisms can be defined for the effects of antibiotics on metabolic syndrome, we can reduce the increasing prevalence and may even develop relevant treatments to reduce or prevent obesity and other related diseases.

In this thesis, we aimed to define the mechanisms through which early life antibiotics impact adverse metabolic outcomes including obesity and imbalance of glucose homeostasis, which may eventually lead to diabetes. We hypothesized that the effects of antibiotic exposure on metabolic syndrome observed in epidemiological research were through the alteration of microbial composition. An altered gut microbiota could programme the development of the intestinal tract, T cell and β-cell functions contributing to metabolic inflammation and modifying glucose and lipid metabolism predisposing to metabolic disease. Since metabolic syndrome is usually triggered by multiple environmental factors and the outcomes of some programming effects will only show in certain conditions, we hypothesized that high fat diet would exacerbate or accelerate the metabolic outcomes of antibiotic programming effects.

The main objectives of this study were as follows:

1. To establish a porcine model of neonatal antibiotic exposure to study the role of microbiota manipulation in obesity and glucose metabolism
2. To examine the alterations of microbial population in the gut induced by early life antibiotic exposure
3. To identify key regulatory genes associated with obesity/diabetes/insulin resistance/pancreatic β-cell development in the intestine and pancreas that were regulated by antibiotic-induced microbial changes
4. To develop a diet induced metabolic syndrome model to be used in future antibiotic exposure studies.

- Chapter 2: Long-Term Impacts of Early Life Antibiotic Exposure on Intestinal Microbiota and Metabolic Disease

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2.0 Introduction

Antibiotic agents have dramatically increased human health and life expectancy since the 1940s. Together with vaccination and other hygienic practices, antibiotics have steadily decreased the morbidity and mortality associated with infectious diseases (Keeney et al., 2014). Antimicrobial agents are widely used and frequently prescribed for infants and children. In some regions, nearly one third of children receive antibiotics in the first 6 months (Trasande et al., 2013). Recent studies revealed that sixty-nine percent of children were exposed to antibiotics within two years of age in the United States and forty-five percent of infants in Canada in the periparturient period (Bailey et al., 2014; Persaud et al., 2014). In addition to concerns related to the development of antibiotic resistance (Zhu et al., 2013; Blair et al., 2015), there is increasing concern that childhood antibiotic exposure may have long lasting

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consequences, such as rheumatoid arthritis, asthma, inflammatory bowel disease and atopic dermatitis (Keeney et al., 2014). In recent years, several epidemiological studies have linked antibiotic treatment in infancy with increased risk of metabolic disease later in life (Ajslev et al., 2011; Azad et al. 2014). However, little experimental evidence for this relationship has been provided, and the cellular and molecular mechanisms are not clearly defined. The mammalian gastrointestinal (GI) tract is home to a diverse and complex ecosystem of commensal gut microbiota that provides essential functions to the host in shaping GI tract, protecting from pathogens, facilitating immune system establishment, providing energy and regulating metabolic pathways by microbial metabolites (reviewed by Fujimura et al., 2010). Early life, especially the first six months, is a critical window for the development of the host, including the colonization of gut microbiota and development of intestine as well as the maturation of pancreatic beta cell and immune system (Vickers et al., 2014). Therefore, interruption of normal colonization by early environmental exposures such as delivery mode, bottle feeding and administration of antibiotics in infancy may disrupt ancient patterns of intestinal colonization and exert durable effects on metabolic and immune profiles via microbial products such as lipopolysaccharides (LPS) and short chain fatty acid (SCFA) (Ajslev et al., 2011).

LPS is a major component of the outer membrane of gram-negative bacteria and elicits strong immune responses in animals. LPS is the main driver of metabolic inflammation and initiates obesity and insulin resistance (Cani et al., 2007; Lassenius et al., 2011). Systemic LPS impairs insulin signaling in β-cells via Toll-like receptor 4 (TLR4) and NF-κB signaling leading to decreased expression of pancreatic and duodenal homeobox 1 (*PDX-1*), a transcription factor critical for both endocrine and exocrine pancreas and in the later differentiation and function of the β-cells (Cani et al., 2007; Amyot et al., 2012). SCFA also play a pivotal role in regulating host metabolism by stimulating production of satiety hormones glucagon like peptide-1 (GLP-1) and peptide YY (PYY) that regulate food intake and energy harvest via

activation of G protein-coupled receptor 41 (GPR41) and GPR43 (Tolhurst et al., 2012; Inoue et al., 2014). SCFA are a subset of fatty acids with less than 6 carbon atoms of which acetate (C2), propionate (C3) and butyrate (C4) are the most abundant (Cook et al., 1998), fermented from partially and nondigestible polysaccharides by anaerobic caecal and colonic microbiota. Administration of SCFA or increasing SCFA concentration by enhancing microbial production in adult animals and people results in improvement in metabolic outcomes (Lin et al., 2013; Byrne et al., 2015), however little is known about their role in the neonate.

Recent data suggest that a complex interplay between gut microbiota and early life programming due to mechanisms such as DNA methylation and post-translational histone modifications (Remely et al., 2014; Kumar et al., 2014). For example, expression of TLR2 and TLR4 is regulated by DNA methylation in intestinal epithelial cells, which depends on the presence of commensal bacteria (Kellermayer et al., 2011; Takahashi et al., 2011). It has also been reported that butyrate epigenetically regulates IAP expression (Hinnebusch et al., 2003). IAP is a gut microbiota-regulating factor that suppresses *E.coli* growth and detoxifies bacterial LPS so as to limit inflammation (Campbell et al., 2010; Kaliannan et al., 2015). It remains to be determined whether the disrupted microbiota induced by the early life environmental challenges exerts long-term programming of metabolic disease development via microbial metabolites.

A growing body of evidence suggests that modifications of epigenome, such as cytosine methylation of DNA, histone post-translational modifications, and micro-RNA, due to the alterations in the *in utero* environment could play a significant role in increasing the susceptibility to metabolic disease later in life (Nolan et al., 2011; Van et al., 2015). More importantly, results from both intrauterine animal models and genome-wide association studies (GWAS) suggest that development of obesity and diabetes is associated with epigenetic modifications of pancreatic

development in early life via transcription master regulators (Sandovici et al., 2013; Sosa-Larios et al., 2015; Xie et al., 2015). In a rat model, development of diabetes after IUGR has been found to be related to hypermethylation of *PDX-1* (Park et al., 2008; Dayeh et al., 2014). In addition, *IGF-2*, which is a key autocrine regulator of β-cell growth and proliferation acting through Irs1/Irs2-PI3K-Akt pathway (Fujimoto et al., 2009), was also identified to be epigenetically regulated in islets from type 2 diabetes (T2D) (Volkmar et al., 2012). It has not been explored whether the programming of these genes is also influenced in the early postnatal period, particularly by the microbial environment.

To provide experimental evidence that antibiotic exposure impacts metabolic outcomes and to explore the underlying mechanisms of how these effects alter the intestinal and pancreatic development, which predispose to the onset of diabetes and obesity, we developed a neonatal pig model of postnatal antibiotic treatment. Remarkably, we found that effects of therapeutic doses of an antibiotic commonly prescribed to children (amoxicillin) on overall microbial composition are limited and microbial communities recovered rapidly after the cessation of antibiotics treatment, yet the metabolic phenotypes in glucose and SCFA metabolism persisted, emphasizing the importance of early life microbial programming in host growth and development later in life.

2.1 Materials and Methods

2.1.1 Ethics Statement

This study was performed according to the guidelines provided by Canadian Council on Animal Care and with approval of the University of Alberta Animal Care and Use Committee (AUP00000922). Pigs used in this study were managed according to approved protocols at the Swine Research and Technology Centre (SRTC), University

of Alberta.

2.1.2 Animals and Experimental Design

Four litters of crossbred (Duroc × Large White/Landrace) piglets (1.42 ± 0.05 kg) born to 2nd or 3rd parity sows, were blocked by sex, weight and litter origin, before being randomly assigned as individual pigs to antibiotic (ANTI) and control (CON) treatments. Two piglets from each litter (1 male and 1 female) were terminated at birth for baseline measurements (postnatal day (PND) 0). The rest of the piglets were given either oral amoxicillin (N = 22) or placebo (N = 23) 30mg/kg/day divided into two doses (8am and 4pm). Treatments were administered continuously for 14 postnatal days starting from birth. Body weight data and fecal samples were taken every week. Pigs were vaccinated as per standard herd protocol, but not exposed to antibiotic outside of assigned treatment.

At PND 21, 8 pigs from each treatment were terminated for sample collection. The rest of the pigs were weaned at 21 ± 1 d of age and moved to an assigned nursery room, penned by litter with unlimited access to feed and water. Nursery pigs were fed with a phase-feeding programme according to SRTC nursery guidelines. At PND 49, 8 pigs from each treatment were terminated for sample collection. The rest of pigs were moved to metabolic pens two weeks before oral glucose tolerance test (OGTT) and housed individually in fully slatted pens (1.2 m × 0.9 m). The pigs were provided with *ad libitum* access to feed and water from a nipple-in-bowl drinker. All animals were housed at a temperature of 22°C to 25°C with a 12-hour dark: light cycle. Pigs were vaccinated with circovirus at weaning, erysipelas and Ileitis at week 7 before OGTT and observed closely at least twice daily. OGTT was then conducted at 7 weeks of age on all the animals (8 pigs from each treatment). Individual body weight and feed disappearance were used for calculation of average daily gain (ADG) and average daily feed intake (ADFI).

2.1.3 Fecal and Intestinal Digesta Sampling

Fecal or intestinal digesta samples were obtained at PND 7, 21 and 49. At PND 7, rectal cotton swabs were collected using sterile dry swabs (BD Falcon™ SWUBE™, BD Canada, Mississauga, ON). The swab was inserted into the rectum and removed gently and stored in −80°C until DNA extraction. At PND 21 and PND 49, digesta samples from ileum (20cm proximal to the ileo-caecal junction) and distal colon (60cm from rectum) were collected during dissection.

2.1.4 Blood Glucose and Plasma Insulin Measurement

Oral glucose tolerance test (OGTT) was performed after an overnight fasting as described (Montelius et al., 2014) with some modifications. Briefly, before OGTT, pigs were allowed an acclimatization period of 2 weeks during which they were gradually introduced to their respective diets and trained in the experimental procedures including being picked up by handlers and adapting to staying in a sling. An hour before OGTT, pigs ears were cleaned and Lidocaine/prilocaine cream (EMLA Cream; AstraZeneca, Mississauga, Ontario) was applied to the skin overlying the marginal veins of the pig ears to reduce pain throughout OGTT. During OGTT, glucose was measured in whole blood with a glucometer by ear pricking (Accu-Check Compact Plus; Roche Diagnostics) over a 3 h period. Fasting plasma glucose (FPG) concentration was measured before OGTT. Animals were then offered a meal consisting of 50g of grounded pregrower fodder mixed with 2g/kg glucose solution. The meal was eaten from a bowl under supervision. After glucose detection, additional 150µl blood samples were collected into Eppendorf tubes by 70µl microhematocrit capillary tubes coated with ammonium heparin (Fisher Scientific) every 15 min from -15 to 60 min, then every 30 min to 180 min for insulin measurement. Samples were centrifuged at 8,000×g for 10 min at 4°C and supernatant

was collected and stored at -80°C until assayed for insulin by ELISA, according to the manufacturer's instructions (Alpco Diagnostics, Salem, N.H., USA). Area under the curve was calculated in accordance with established methods (Wolever, 2004).

2.1.5 Animal Euthanasia and Sampling

Two pigs (1 male and 1 female) from each litter (n=8) were terminated as baseline at PND 0. At PND 21 and 49, two pigs per treatment per litter were euthanized for sample collection. General anesthesia was induced in pigs with an injection of 16mg/kg ketamine HCl (Ketalean; Bimeda-MTC, Cambridge, ON, Canada), 2.2 mg/kg xylazine (Rompun; Bayer Inc., Toronto, ON, Canada), and 6mg/kg azaperone (Stresnil; Janssen Pharmaceutica, Beerse, Belgium). Afterwards, 5% isoflurane (Isoflo, Abbott Laboratories Ltd., Saint-Laurent, Quebec, Canada) was provided using a face mask. Blood samples were collected aseptically by jugular venous puncture into endotoxin-free blood collection tubes coated with EDTA (S-Monovette, Sarstedt, Canada) and centrifuged promptly (2,000 × g for 10 min at 4°C) and plasma separated and stored at -20°C until analysis. Pigs were immediately exsanguinated.

After exsanguination, the abdominal cavity of each pig was opened and pancreas was collected for pancreatic islets isolation and culture (stored in ice cold Hanks' Balanced Salt Solution [HBSS] supplemented with 0.2% BSA, 25mM HEPES, 100 mg/L L-glutamine and 0.35g/L $NaHCO_3$; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), immunohistochemistry (10% formalin, Fisher Scientific) and gene expression (snap frozen in liquid nitrogen). The entire small and large intestines were subsequently removed. The ileum (20cm proximal to the ileo-caecal junction), proximal colon and distal colon were carefully dissected. Digesta from the ileum and distal colon for microbial analysis were aseptically collected and snap frozen in liquid nitrogen until storage at -80°C. Caecal contents were collected into sterile plastic containers and stored at -80°C for SCFA measurement. Intestinal samples were

opened longitudinally and rinsed with ice cold PBS. Digesta was removed and mucosal samples were collected from these tissues by scraping the mucosal lining with a glass slide. These samples were immediately snap-frozen in liquid nitrogen and then stored at -80°C for subsequent RNA isolation and enzyme activity measurement.

2.1.6 Pancreatic Islet Isolation and Culture

Isolated pancreatic islets of Langerhans were obtained by collagenase digestion as described (de Haan, 2004) except the duration for collagenase digestion was adjusted according to digestibility of tissue as shown in Table 1.1. After digestion, the samples were filtered through 160μm nylon filter and rinsed with HBSS supplemented with 0.2% BSA (Salvalaggio et al. 2002), then centrifuged. Each pellet sample was enriched for islets by means of a 27%, 23%, 14% dextran density gradient centrifuged at 1,500 × g for 15 min at room temperature. Islets were harvested from the 14%-23% interface. After washing steps, islets were suspended in HBSS with 0.2% BSA and separated from exocrine tissue by hand picking under a dissection microscope (Fritschy, 1992). Islets were cultured for 24 hours in Petri dishes containing CMRL1066 (Sigma) with 10% Fetal Bovine Serum (FBS) at 37°C in humidified air containing 5% CO_2.

2.1.7 Glucose-stimulated Insulin Secretion Assays

Cultured islets were washed with Dulbecco's modified Eagle's medium (DMEM; Gibco, Burlington, ON, Canada) with 0.1% BSA. To measure insulin release, quadruplicate samples of 3 islets/vial were incubated in DMEM with appropriate glucose concentrations (2.8, 5.5, 11, 16.5, 22 mM) for 90 min at 37°C gassed with 95% O_2 and 5% CO_2. Supernatants were retained and insulin remaining in the islets was extracted with 3% acetic acid, and then stored at −20°C for insulin radioimmunoassay (RIA) (Yang et al, 2015). Total islet insulin content was calculated by adding insulin

secreted into supernatant plus the remaining in the islet pellet, as determined by RIA. From this, the percentage of total insulin secreted was calculated for each data point to eliminate variance caused by islet size. Insulin release index was calculated as the ratio of insulin percentage release in response to 16.5 mM glucose versus 2.8 mM glucose and 22 mM glucose versus 2.8 mM.

2.1.8 Immunohistochemistry

Pancreas tissue was harvested prior to islet isolation by taking a small piece of tissue and fixing in buffered formalin for 24 h. The tissues were dehydrated and embedded in paraffin using standard procedures and 5 μm sections cut and affixed to glass slides. Following dewaxing, sections were treated with 10% H_2O_2 (Sigma) to inactivate endogenous peroxidase, and then blocked using 5% normal serum complementary to the respective secondary antibody. For insulin staining, the sections were then incubated at room temperature for 3 hours with guinea pig anti-insulin antibody (Dako, Burlington, ON, Canada) diluted 1:200 as primary antibody. Following stringent washing twice in PBS, secondary antibody rabbit anti-guinea pig IgG (whole molecule)-peroxidase antibody (Sigma, A5545; 1:200) was applied for 2 h at room temperature. As for glucagon, primary and secondary antibodies were rabbit anti-glucagon (Millipore, Billerica, MA, USA; 1:200) and goat anti-rabbit HRP (Sigma, 1:200) with the same incubation steps as insulin. Slides were coverslipped in Clarion (Sigma). Digital photomicrographs were obtained using a Zeiss fluorescence microscope and Axiovision 4.7 software (Carl Zeiss Microscopy GmbH, Jena, Germany). The β-cell area expressed as a percentage of the total pancreas area was then calculated.

2.1.9 Circulating Endotoxin Detection

Plasma endotoxin concentrations were measured by a kinetic fluorescent assay using

the PYROGENT-5000 kit (Lonza, Mississauga, Canada). All procedures were performed under non-pyrogenic conditions. Briefly, the plasma samples were diluted 5 × in non-pyrogenic water and were heat inactivated at 70°C for 30 min. A 100 µl aliquot of the samples and standards were added to a 96 well round bottom plate and incubated at 37°C for 10 min. After incubation, 100µl of the reconstituted PYROGENT™-5000 Reagent assay buffer was added to the plate and a kinetic reading was taken up to 1h at 37°C. Thereafter, the relative fluorescence unit for each well was determined (absorbance 340nm). The concentration of the endotoxin was interpolated from the standard curve constructed from the standards and corrected for sample dilution.

2.1.10 IAP Activity Detection

Alkaline phosphatase activity in the ileal mucosa was measured by SensoLyte® pNPP Alkaline Phosphatase Assay Kit Colorimetric (AnaSpec, product #72146, USA). The assay was performed according to the manufacturer's protocol for biological samples. Specifically, approximately 40mg of frozen ileal mucosal samples was thawed in 1ml of ice-cold homogenizing buffer (provided by the kit) and homogenized for 1min with glass beads (1.0mm dia, BioSpec Products, Bartlesville, USA). The resulting homogenate samples were measured for analyses of protein content and enzyme activity kinetics for calculating mucosal IAP. The samples were diluted 100 times for enzyme detection and 10 times for protein concentration measurement. IAP activity was normalized to protein concentrations of total homogenate, as measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Ottawa, ON, Canada). The intensity of color corresponding to the alkaline phosphatase activity was read using SpectraMax M3 Microplate Reader (SpectraMax M3, Sunnyvale, CA) and the absorbance was measured at 405 nm.

2.1.11 SCFA Measurement

Caecal content samples were analyzed for SCFA profile using gas chromatography with a modified method (Jha et al., 2015). Briefly, 1g of caecal content was weighted and mixed with 4ml of 25% phosphoric acid. Samples were vortexed thoroughly and centrifuged at 3,500 × g for 10 min at 4°C. Supernatant was transferred to 1.5ml tubes and centrifuged at 15,000 × g for 10 min at 4°C. The supernatant was then filtered using 0.45μm filter units (Fisher). 0.8 ml of filtered supernatant was added into a GC vial (12 × 32mm, Thermo Scientific) with 0.2 ml of internal standard solution containing 24.5 mmol/L isocaproic acid and vortexed. Samples were injected by an autosampler (Model 8400; Varian Inc., Walnut Creek, CA) into a Stabilwax-DA column (30 m × 0.53 mm i.d. × 0.5 μm film thickness; Restek Corporation, Bellefonte, PA) on a Varian gas chromatograph (Model 3800; Varian Analytical Instruments, Palo Alto, CA). Samples were run at a split vent flow of 20 mL/min with a column temperature of 80°C for 0.0 min, and then increased to 210°C at a rate of 45°C/min and held for 5.11 min at 210°C. The injector temperature was 250°C, and the detector temperature was 250°C. Peak integration was evaluated using Galaxie Software (Varian Inc., Palo Alto, CA). Total SCFA concentration was calculated by the sum of all the detected short chain organic acids, expressed as μmol/g digesta. Final results were normalized according to the weight of original sample used.

2.1.12 RNA Isolation and Quality Control

Total RNA from the pancreas, ileum and proximal colon whole tissues was extracted using the GeneJET RNA Purification Kit (Thermo Scientific). Frozen tissues were ground to powder with chilled mortars and pestles in liquid nitrogen and homogenized with motorized tissue grinder (Fisher Scientific Ltd, cat. no.03392106) in lysis buffer provided by the kit as per manufacturer instructions. On column DNase (RNase-free DNase set; QIAGEN) was applied to eliminate genomic DNA contamination in the RNA samples. All the isolation steps were done in a RNase-free environment to

minimize RNA degradation. Concentrations and purity of RNA samples were determined spectrophotometrically by NanoDrop 2000c (Thermo Scientific). The quality was determined using a ratio of absorbance at A_{260}/A_{280} with a minimum ratio of about 2. RNA quality was also verified with gel electrophoresis (RNA loading dye, Thermo Scientific) by a method with minor modification. If the ratio of 28S and 18S rRNA bands is close to 2:1, the isolated RNA can be considered as intact. Samples with strong bands of 5SrRNA or other small RNAs and weak bands of 28SrRNA were regarded degraded (Aranda et al., 2012).

2.1.13 Quantitative RT-PCR

To quantify mRNA expression, a combined technique of reverse transcription PCR and (qRT-PCR) quantitative PCR was used. Extracted RNA was firstly reversely transcribed (1µg) with Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) and then used as a template and analyzed by StepOne software v2.3 on the StepOnePlus real-time PCR System (Applied Biosystems, ON, Canada). Ileal cDNA and pancreatic cDNA were diluted 1:8 and 1:4, respectively, prior to qRT-PCR. A two-step thermal cycling protocol was performed as follow: Initial denaturation, one cycle at 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s and annealing or extension temperature for 30 s. Primers used in the qPCR were designed in Beacon Designer 7.9 by using sequences obtained from the ENSEMBL pig database. A PCR reaction was conducted to test the amplification products and annealing temperature of the primers. Specificity of the amplification was verified by melt curve analysis and evaluation of efficiency (90%-110%) of qPCR amplification and no reverse transcriptase control (NRT) was run as negative control for genomic DNA contamination. The primer sequences are listed in Table 2.2. Samples were analyzed in duplicate and mRNA expression and *GAPDH* used as the internal control. qRT-PCR data were analyzed using relative quantification and the comparative CT ($2^{-\Delta\Delta CT}$) method as described previously (Livak et al., 2001). Relative quantification

values are presented as fold changes listed below:

Fold change = $2^{-\Delta\Delta CT}$ = [(C_T genes of interest – C_T internal control) samples treated with antibiotics – (C_T genes of interest – C_T internal control) samples not treated with antibiotics]

2.1.14 DNA Extraction

For microbial composition analyses, total DNA from ileal and distal colon contents from PND 21 and 49 was extracted with the QIAamp DNA Mini Stool Kit (Qiagen, Inc. Mississauga, ON, Canada) following the manufacturer's instructions, with the addition of a bead beating step (FastPrep instrument, MP Biomedicals, Solon, OH). Total DNA was isolated from swab samples and intestinal contents with a DNA concentration and quality were determined spectrophotometrically by NanoDrop 2000c (Thermo Fisher Scientific, Inc., Ottawa, ON, Canada). Extracted DNA was diluted to 20ng/µl for PCR amplification.

2.1.15 Microbial Analysis

2.1.15.1 16S rRNA gene amplification and sequencing

The hypervariable regions (V1 to V3) of the bacterial *16S rRNA* gene were amplified with nucleotide-barcoded primer pairs 27F: 5′-AGAGTTTGATCMTGGCTCAG-3′ and 519R: 5′- GWATTACCGCGGCKGCTG-3′. The forward primer contained Roche/454 Titanium adaptor A (CCATCTCATCCCTGCGTGTCTCCGACTCAG) and unique 10-bp barcodes, the reverse primer contained adaptor B (CCTATCCCCTGTGTGCCTTGGCAGTCTCAG). DNA amplification was carried out in 20-µl reaction volumes containing 0.2µl Phusion high-fidelity DNA polymerase (Thermo Scientific.), 4µl of 5 × HF buffer, 0.4µl 10mM dNTPs, 1 µl of the extracted template DNA and 1 µl each of forward and reverse primers (10ng/µl). The PCR for

each method was carried out in an S1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) with the following parameters: initial denaturation at 98°C for 1 min, followed by 35 cycles of 98°C for 10 s, 59°C for 30s and 72°C for 30s, with a final extension at 72°C for 7 min. Following PCR amplification, Triplicate DNA amplification products were mixed and run at 100 V for 1 hour by gel electrophoresis on a 1% agarose gel (SYBR Safe stain, Invitrogen). The bands corresponding to bacterial *16S rRNA* was excised and gel-purified (QIAquick gel extraction kit, Qiagen, Valencia, CA). Each amplicon (100 ng) was pooled and pyrosequenced using a 454 Titanium platform (Roche, Branford, CT).

2.1.15.2 Microbiota assessment

Sequence data that passed Roche's quality thresholds were processed according to the mothur 454 SOP (Schloss et al., 2009, 2011) accessed on June 16, 2015. Sequences were provided via .fasta file and the sequence quality was denoted with a .qual file. Barcodes were trimmed and quality sequences were obtained by removing sequences containing ambiguous bases, quality read length <200 bases. Sequences passing quality filter were aligned to the silva bacterial reference alignment. Sequences were clustered based upon 0.97 similarity using UClust into operational taxonomic units (OTUs) and hypothesis testing were performed with normalized data in mothur. The Shannon Diversity Index (SDI), a measurement of within-sample (alpha diversity) community diversity was used to ascertain differences in alpha diversity based on antibiotic exposure status. Alpha diversity measurement was calculated with mothur and significance was measured using a parametric t-test for comparison of microbial composition of all antibiotic treated or non-treated pigs. UniFrac unweighted distance matrices were calculated from the OTU phylogenetic tree for beta diversity analyses.

2.1.16 Statistical analysis

Data were expressed as mean ± SEM. Data from OGTT, LPS activity and growth rate were analyzed by univariate analysis of variance (ANOVA) to examine associations between variables. Main effects were analyzed using Bonferroni post hoc tests. Glucose and insulin data were converted to area under the curve (AUC) values by the trapezoidal method. Comparisons between the amoxicillin treated and the placebo treated groups were performed using independent-samples Student's t test to evaluate the associations of continuous variables after normality tests. We defined $P \leq 0.05$ as significant and a P value between 0.1 and 0.05 as a trend. Data were analyzed using GraphPad Prism v. 6.02 (La Jolla, CA) and IBM SPSS Statistics, version19.

2.2 Results

2.2.1 Early Antibiotic Exposure Does Not Alter Body Weight

We exposed newborn piglets to human therapeutic doses of amoxicillin orally for two weeks. To examine whether early antibiotic exposure affected piglet growth traits, body weight was recorded weekly. Birth weight was similar in both groups, CON (1.338 ± 0.058 kg, N = 16) and ANTI (1.307 ± 0.074 kg, N = 14). Results show that antibiotic exposure did not affect overall growth phenotypes throughout the experiment (Figure 2.1). By PND 49, body weight in CON and ANTI was 16.37 ± 0.47 kg and 15.96 ± 0.66 kg respectively. Feed disappearance was recorded from PND 34 to 48 and tended to be greater in ANTI pigs. Although it was not quantified, it was observed that the ANTI pigs wasted more feed, therefore feed disappearance may not accurately reflect feed intake. Body composition was not assessed, so it is unknown whether there was an effect of treatment on adiposity.

2.2.2 Early Life Antibiotic Alters Glucose Metabolism

Although obesity and diabetes are highly connected, there are several other factors that are recognized as major components that predispose diabetes, including pancreatic dysfunction, insulin resistance and increased hepatic glucose production, which may appear without the obese phenotypes (Carnethon et al., 2012). To examine the glucose metabolism in pigs treated with amoxicillin, an OGTT was performed at PND 49. In a pilot study it was confirmed that blood glucose sampling caused minimal stress as indicated by stable blood glucose levels over time in the absence of glucose challenge. The OGTT was used to assess how quickly the given glucose is cleared from the blood and glucose concentration returns to fasting levels. It is usually used to test for diabetes, insulin resistance and impaired beta cell function. Antibiotic exposure in the first two weeks was shown to have significant effect ($P < 0.05$) on host glucose control during OGTT by two-way ANOVA analysis (Figure 2.2). No effect of litter was observed, therefore it was excluded from the analysis. The ANTI group peaked at 60 min and glucose level was significantly higher ($P < 0.05$) than that of CON group. Area under the curve (AUC) of glucose concentration during OGTT also showed a trend ($P < 0.1$) toward elevated glucose concentration, thus lower glucose tolerance, in the ANTI group. There was no significant difference in insulin secretion between the two treatments in the first 45 min during the OGTT. Therefore, with similar insulin levels, higher glucose concentration in ANTI group suggests delayed glucose uptake from the blood (Figure 2.2).

2.2.3 Metabolic programming: Early Life Antibiotic Alters Pancreatic Development

Reduced glucose tolerance is usually associated with pancreatic islet dysfunction and is considered an early predictor of diabetes (Kasuga, 2006). To further examine the early antibiotic treatment on pancreatic β-cell function and to test whether glucose intolerance during OGTT at PND 49 was caused by impaired insulin signaling from pancreatic islets, GSIS assay was conducted on isolated islets at PND 21 and PND 49

(Figure 2.3). At PND 21, isolated islets from pigs treated with antibiotics had higher insulin content than that of CON group ($P < 0.05$). In addition, islets from ANTI were more responsive to glucose as indicated by insulin release index ($P < 0.05$). Both insulin content and release index are indicators of pancreatic ability in producing insulin when challenged with high glucose solution. At PND 49, there was no difference in insulin content or insulin release index in isolated islets, indicating comparable β-cell function in both groups at this age. From PND 21 to PND 49, there was a significant increase in insulin content ($P < 0.05$) and 45% increase in insulin release index in CON islets. In contrast, with these changes in CON, a lower value in both measurements was observed in the ANTI group.

To assess insulin-positive (β-cell) and glucagon-positive (α-cell) area in two groups at PND 21 and PND 49, paraffin fixed samples were sectioned and stained for insulin and glucagon (Figure 2.4). Immunofluorescence showed that the fractional islet insulin-positive area tended to be higher in ANTI group at PND 21 ($P = 0.08$). However, percentage of β-cell area decreased significantly ($P = 0.03$) from PND 21 to PND 49 in antibiotic treated pigs while pigs treated with placebo did not show much difference (Figure 2.5), which means there was more insulin in ANTI pancreas at PND 21 and the same at PND 49 when compared with control. The results are in accordance with GSIS assay results. The same trend was observed when staining for glucagon was examined (Figure 2.4). These data indicate a clear alteration in beta-cell development with respect to differentiation, proliferation or apoptosis when challenged with antibiotic treatment in early life.

To further explore the potential mechanisms that led to the β-cell dysfunction, genes that are involved in pancreatic development, insulin secretion and β-cell function were analyzed by qRT-PCR in whole pancreas at PND 21 and PND 49 (Figure 2.6). The *PDX-1* transcription factor is known to play an indispensable role in development of both the endocrine and exocrine pancreas and in the later differentiation and secretory

function of the β-cell. Particularly, *PDX-1* transactivates the insulin gene and other genes involved in glucose sensing and metabolism (Kaneto et al., 2007). At PND 21, relative *PDX-1* mRNA levels in ANTI group were 30% lower than that of the CON group (P = 0.02). This difference was not observed at PND 49. As an important pancreas marker that protect islets from onset of type 2 diabetes, lower expression in *PDX-1* indicates altered programming induced by amoxicillin exposure at PND 21. We also measured the expression *IGF-2* because it is involved in β-cell regeneration and proliferation (Zhou et al., 2012) and has been reported to be maternally imprinted and epigenetically regulated in newborns exposed to antibiotics (Vidal et al., 2013). Consistent with GSIS results, expression of *IGF-2* showed a 1.5-fold increase in expression relative to CON at PND 21 (P = 0.06). In contrast, lower expression of *IGF-2* was observed in amoxicillin treated pigs than those received placebo at PND 49 (P = 0.03). There was a non-significant trend for higher *INS* (gene that encodes insulin) expression in ANTI (P = 0.09) in pancreatic tissues at PND 21. At PND 49, there was no difference in *INS* expression between the two groups. The *INS* expression agreed with the results in GSIS assay and immunofluorescence.

2.2.4 Amoxicillin Alters the Composition of Intestinal Microbiota

To examine direct effects of therapeutic amoxicillin on gut microbiota in pigs and seek the relationships between the metabolic changes and gut microbial composition, we extracted DNA from swab fecal samples at PND 7 and ileal and distal colonic content at PND 21 and PND 49. The extracted DNA was subjected to 454 pyrosequencing, yielding 1907 ± 86 sequences per sample that passed quality filtration and removal of chimeras. The sequences were analyzed at the community level and at multiple taxonomic levels. Examining the overall community structure revealed that a global change in composition was not induced by antibiotic treatment at any time point in any tissue as indicated by ANOVA ($P \geq 0.27$). As expected there was a significant effect of sampling location (ileum vs distal colon) and sampling time (PND 7, 21 and 49) on community composition (ANOVA, $P < 0.001$) and as can be

visualized by PCoA plot (Figure 2.8). In general, there was no significant difference in the two groups at phylum level, however, during the course of antibiotic treatment (PND 7) there were some changes in specific genera. A substantial increase in the family Enterobacteriaceae was observed in amoxicillin treated pigs compared with CON at PND 7 ($P < 0.05$), which was largely represented by a single OTU classified as *Escherichia/Shigella* (6.0% and 1.2%). Another family that significantly differed in ANTI and CON was Erysipelotrichaceae ($P < 0.05$) (Figure 2.7). The final notable change in microbial population at PND 7 in response to amoxicillin treatment was the increase in an OTU identified as *Mitsuokella jalaludinii*, which was undetectable in all CON samples, but detected in 8 of 11 ANTI pigs and represented a mean of 0.17% of 16S rRNA gene reads. Importantly, all of the differences observed at day 7 were lost after antibiotic treatment ended (PND 21 and 49). The composition of gastrointestinal microbiota was assessed using alpha and beta diversity measurements. There was no effect of antibiotic treatment on alpha diversity as indicated by the inverse Simpson-diversity index. However, there was a trend for reduced beta diversity in antibiotic treated pigs ($P = 0.06$) (Figure 2.9).

2.2.5 Changes of LPS and Host Responses to Early Life Antibiotic Exposure

It is well accepted that higher circulating LPS is associated with inflammation in obesity and diabetes (Cani et al., 2008, Zhao et al., 2013). To determine whether antibiotic induced microbial change leads to altered LPS concentration, we measured LPS in circulating blood at PND 21 and PND 49. The antibiotic treatment showed a trend to increase LPS activity in the amoxicillin treated pigs ($P = 0.06$) over time by two-way ANOVA analysis (Figure 2.10). We also measured the capacity of the host to detoxify LPS by measuring IAP activity and gene expression in ileum mucosa at PND 21 and PND 49 (Figure 2.11). There was no significant difference in both groups at PND 21. However, at PND 49, there was a trend ($P = 0.08$) of lower IAP in amoxicillin treated pigs than that of CON group. With higher LPS concentration and lower IAP, pigs treated with antibiotic were more likely to be challenged by LPS

induced systemic inflammation. However, when comparing gene expression of *IAP* by whole ileum tissue extracted RNA, the difference no longer existed.

2.2.6 Early Antibiotic Exposure Alters Later Gut Microbiome SCFA Metabolism

Because of the central role of SCFA in colonic metabolism and host-microbe signaling, we measured the concentration of SCFA in caecal content at PND 21 and PND 49 (Figure 2.12). Direct measurement of SCFA demonstrated a decrease in total SCFA concentrations ($P = 0.08$) and especially the most abundant SCFA, acetate ($P < 0.05$) in ANTI group. At PND 49, the difference between the two groups was not observed. To examine whether reduced SCFA caused by antibiotic treatment has a further effect on host metabolism, we performed qRT-PCR of *GPR41* at PND 21 and PND 49. At PND 21, there was no obvious difference in *GPR41* expression. However, at PND 49 *GPR41* expression was 2-fold higher in CON than ANTI ($P < 0.05$). Western blotting for GPR 41 was attempted, but it did not work.

2.3 Discussion

The global pandemic of obesity and T2D is largely caused by westernized diet and sedentary lifestyle, which contribute to an increase in dietary energy intake and reduction in physical energy expenditure. However, growing attention is being paid to the role of developmental plasticity and alterations in metabolic outcomes resulting from early life microbial disruption induced by antibiotic treatment. Although several epidemiological studies have correlated antibiotic exposure in infancy with increased risk of metabolic disease later in life (Trasande et al., 2013; Bailey et al., 2014; Saari et al., 2015), this is the first pig model that experimentally provides evidence showing that therapeutic antibiotic amoxicillin alters islet development, with implications on metabolic regulation.

By using a pig model for human infants to assess metabolic phenotypes, we show that therapeutic doses of amoxicillin in the early period (PND 1-7) induce glucose intolerance in later life (PND 49). The loss of glucose regulation suggests compromised insulin secretion from pancreatic β-cells or insulin signaling in peripheral tissues, or both. Results from GSIS assay in isolated islets and immunostaining of insulin in pancreatic sections indicated that the reduced glucose tolerance at PND 49 in ANTI could be associated with the altered β-cell development and hyper-functioning of pancreatic islet at weaning though it becomes less different between the groups at PND 49. Although the main glucose transporter (*GLUT2*) is required for glucose-stimulated insulin secretion in β-cells and inactivation of *GLUT2* was associated with suppressed GSIS (Thorens et al., 2014), the impaired glucose regulation in this study was unlikely due to *GLUT2*-dependent glucose sensing since the gene expression of *GLUT2* in β-cells was not significantly different in two groups (data not shown). The underlying mechanisms remain to be determined. Notably, at PND 21, islets from antibiotic treated group showed higher insulin production capacity when challenged with high concentration of glucose in GSIS assay and also had more insulin positive area in pancreatic section compared with placebo treated pigs. However, after 4 weeks of growth and development in a normal pig raising environment, the differences were not observed between the two groups. Remarkably, islets from antibiotic exposed pigs showed significant progressive reduction in β-cell area and their insulin secretion as indicated by comparing the results from weaning to four weeks later. The observed metabolic phenotypes provided evidence for the programming of early life antibiotic exposure on host development.

It has been appreciated that substantial remodeling of the endocrine pancreas occurs in neonatal life, in which pancreatic β-cells undergo apoptosis and new islets generate from the ductal epithelium, which is associated with a transient decreased growth rate of islets at weaning in rodents, pigs and humans (Scaglia et al., 1997; Hill et al., 2000; Bock et al., 2003; Kassem et al., 2000). Self-replication is low in adults, which makes

the pancreatic islets unable to regenerate significantly following extensive tissue injury or chronically increased metabolic demands (Bouwens et al., 2005). Beta cells are highly dynamic and resilient and will compensate to cope with insulin demand when challenged with high circulating glucose (Cerf, 2013). Therefore, important changes taking place at the level of β-cell maturation and perturbation before weaning may exert long term consequences. Progressive loss of function in ANTI pigs suggests that islets regressed from hyper-functioning at PND21 to "normal" functioning at PND49, making them prone to glucose intolerance. Although our pig model did not show symptoms of type 2 diabetes compared with high-dose streptozotocin induced pig models (Grüssner et al., 1993), early pre-diabetic symptoms of β-cell dysfunction, which is present prior to the development of type 2 diabetes (Kahn, 2003), showed the strong programming effects of antibiotics on pancreatic development when exposed for only two weeks in the early period.

Although, the alteration of glucose metabolism was observed in ANTI group, there was no difference in growth rate between two groups. Feed disappearance in ANTI pigs was observed to be higher than control pigs, however, it was noted that ANTI pigs wasted more feed compared with CON, suggesting feed disappearance could not be used as an accurate measure of feed intake. Interestingly, recent studies have linked early life programming of microbiota with neurodevelopment and normal functioning of the brain-gut axis (O'Mahony et al., 2015; Jašarević et al., 2015). The feed wasting behavior may be related with changes in neuron development; however this relationship was not further explored. In addition, it was demonstrated previously that sub-therapeutic doses of antibiotics significantly increased adiposity and altered hepatic metabolism of fatty acids and lipids in mice (Cho et al., 2012). It will be of great interest to assess whether early life antibiotics increase adiposity and alter lipid metabolism in pigs later in life.

Under normal conditions, the pre- and postnatal development follows a certain routine

pattern as a consequence of multiple environmental and innate developmental factors. When the normal development is disrupted by external stimuli, early life antibiotic exposure as an example, unpredictable consequences may be observed. The lower expression of *PDX-1* in the ANTI pigs at weaning may explain the alteration of islet cell development as the dysfunction of β-cells is usually induced by the dysregulation of critical transcriptional factors and other related crucial metabolic pathways (Guo et al., 2013; Fu et al., 2013). As *PDX-1* plays a central role in β-cell function and survival, the decreased expression of *PDX-1* could result in dysregulation of downstream genes (Fujimoto et al., 2009). In an IUGR rat model, *PDX-1* mRNA levels were reduced by 50% and associated with alterations in histone modifications (Park et al., 2008). It was also reported that maternal nutrition deficiency could increase the duration of β-cell cycle in offspring (Petrik et al., 1999). Similarly, pancreatic islets from type 2 diabetic patients showed increased DNA methylation and decreased expression of *PDX-1* (Yang et al., 2012). However, as there was no difference of *PDX-1* mRNA at PND 49, it seems that the effect of antibiotic on *PDX-1* expression were transient although the phenotypes persisted. In contrast to *PDX-1*, high expression of *IGF-2* and *INS* at PND 21 and lower expression at PND 49 were observed in antibiotic treated pigs in this study, which suggest that pancreas of these pigs, might undergo earlier neogenesis before CON group that makes them prone to glucose intolerance. The gene expression results coincide with our data from GSIS and immunostaining of insulin. As discussed above, islets undergo apoptosis and neogenesis postnatally. The timing of apoptosis is associated with a loss of *IGF-2* expression (Petrik et al., 1999). In addition, it was only recently shown that overexpression of *IGF-2* in β-cells could lead to β-cell dysfunction and makes islets more vulnerable to β-cell damage (Casellas et al., 2015). Therefore, our observation of higher expression of *IGF-2* in ANTI at weaning indicates an alteration to the normal remodeling. In ongoing studies it will be important to determine whether the changes in *PDX-1* and *IGF-2* gene expression are associated with gene methylation or other epigenetic modifications and the factors that caused overexpression of *IGF-2* at

weaning.

To explore how the antibiotics might alter metabolic phenotypes, we examined the microbial composition in fecal samples. Although there was no global change in composition of microbiota between two groups, much higher percentage of Enterobacteriaceae and Erysipelotrichaceae was observed in ANTI pigs seven days after continuous antibiotic administration. The global microbial resilience to antibiotic treatment coincides with findings from a mouse model for antibiotic exposure and another swine study that showed antibiotic treatment to sows lead to transient changes in the microbiota of offspring. However, the changes of microbiota in antibiotic treated animals were not consistent in the two studies (Cho et al., 2012; Arnal et al., 2015). Enterobacteriaceae is a large family of gram-negative bacteria, consisting of both commensals and pathogens like *Salmonella* and *Escherichia coli*, which contribute to the enteric pool of LPS that can reach the circulation. Elevated numbers of Enterobacteriaceae abundance has previously been correlated to obesity development (Conterno et al., 2011) and elevated LPS levels were reported to initiate obesity and insulin resistance (Cani et al., 2007). Notably, many members of Enterobacteriaceae carry resistance genes including genes that encode for extended-spectrum β-lactamases which attack β-lactam ring (Paterson et al., 2006). This may explain why treatment of amoxicillin (a member of β-lactam antibiotics that is frequently prescribed to children worldwide) specifically increased percentage of Enterobacteriaceae. Increase in abundance of Erysipelotrichaceae was observed in a diet-induced obesity model (Turnbaugh et al., 2008) and a decrease was observed after gastric bypass surgery in obese individuals in another study (Zhang et al., 2009), suggesting the involvement of this bacteria family in obesity development or as a consequence of obesity. It was previously confirmed that *in utero* low-dose penicillin exposure perturbed the microbiota in mice transiently and induced alterations in host metabolism and adiposity. Yet, exposing to antibiotics only at weaning did not lead to obesity or loss of glucose homeostasis. More importantly, the growth-promoting

phenotype was transferable in germ-free mice received the antibiotic selected microbiota, suggesting the microbiota, not antibiotics, play a causal role (Cox et al., 2014). Consistent with these findings, microbiota differences observed during antibiotic exposure were lost within a week of antibiotic removal. In summary, amoxicillin exposure does not change overall microbial composition but specifically enriches antibiotic resistant microbes. Furthermore, the subtle changes in the gut altered the functionality of the whole microbiota as indicated by reduced SCFA at an early age. These changes in early life impact pancreatic development and may alter metabolic outcomes later in life despite microbiota recovery.

Changes in microbiota are usually associated with altered microbial metabolites. Low-grade inflammation is commonly observed in obese and diabetic patients. Cani et al. demonstrated that changes of microbiota in HFD induced obese and diabetic mice are associated with endotoxemia and gut permeability (Cani et al., 2008). During bacterial infection, hyperglycemia is frequently observed (Nguyen et al., 2014). It was previously demonstrated that both acute and continuous infusion of LPS significantly altered the glucose metabolism (Nguyen et al., 2014). Higher level of LPS in antibiotic treated pigs suggested more challenges to the immune system, which may trigger β-cell failure and insulin resistance due to tissue inflammation (Donath et al., 2009). It is to be determined how the higher LPS level in the blood influences pancreatic development and β-cell function.

IAP functions to reduce excessive inflammatory responses to the resident intestinal microbiota by detoxifying bacterial LPS and prevents metabolic syndrome in mice (Bates et al., 2007; Kaliannan et al., 2013). It was previously shown that intestinal alkaline phosphatase gene was induced by circulating LPS derived from gram-negative bacteria (Yang et al., 2012). It was shown in a pig model for *in utero* antibiotic exposure that ileal IAP was transiently reduced in offspring at PND 14 born to amoxicillin treated sows with no treatment differences thereafter, which suggests

that early programming of metabolic syndrome may happen even before weaning period (Arnal et al., 2014). Another study also showed that offspring born to antibiotic-treated sows displayed lower jejunal IAP activity at 6 months than that in controls (Mroz et al., 2011). Although there was no difference between two groups at PND 21 in our study, the lower IAP activity in ANTI pigs at PND 49 was observed, which suggests the failure of host defense system when facing the LPS challenge. The differences in time points showing transient IAP activity reduction in the studies may be due to the differences in amoxicillin dosage and the antibiotic administration method as we treated new born piglets directly while they administered antibiotics to the sows. There was no difference in *IAP* gene expression between the treatments in our study. This could be due to the different tissue types used for enzyme activity (ileal mucosal scraping) and gene expression (ileal whole tissue) or post-transcriptional regulation.

In addition to LPS, microbes may also regulate host metabolism through SCFA production. We observed lower SCFA levels in caecal contents in antibiotic treated pigs at PND 21. SCFA have been shown to protect against diet-induced obesity by regulating gut hormones GLP-1 and PYY in response to activation of GPRs. GLP-1 is known to inhibit β-cell apoptosis and stimulate insulin-secreting β-cell proliferation and differentiation (Buteau et al., 2003; Brubaker et al., 2004). Therefore, reduced acetate and total SCFA in ANTI at PND 21 indicate higher risk of obesity and diabetes development. In addition to the protective role as signaling molecules, SCFA provide 5-10% energy for healthy people (Fernandes et al., 2014), absorbed by colonocytes through passive diffusion of undissociated SCFA and active transport of dissociated SCFA anions (Sellin et al., 1999). The two main transporters of SCFA on colon cells, monocarboxylate transporter 1 (*MCT1*) and *MCT4*, were not differentially expressed between ANTI and CON pigs at PND 21 and 49, indicating the difference in metabolic changes were unlikely due to SCFA transportation. Instead, lower expression of *GPR41* was seen in pigs treated with amoxicillin at PND 49.

Interestingly, although both GPR41 and GPR43 are SCFA receptors, gene expression of *GPR43* did not differ in two groups, suggesting involvement of *GPR41*-dependent metabolic pathways. It has been demonstrated that SCFA propionate regulates sympathetic nervous system exclusively via GPR41 and impacts on energy expenditure in animal models (Kimura et al., 2011). It will be of great interest to determine whether the slightly decreased propionate and the significant lower expression of its receptor influence the energy expenditure in ANTI group.

In human studies, it was recently shown in obese and diabetic patients that there was a significant negative correlation between body mass and *GPR41* methylation (Remely et al., 2014). Expression of *GPR41* was found to be up-regulated by SCFA in animal models (Lu et al., 2015). However, it remains unknown whether the early difference in SCFA programming altered *GPR41* expression at a later time. In contrast, it was reported that in a mouse model, sub-therapeutic doses of antibiotics resulted in an elevated ratio of Firmicutes to Bacteroidetes and increased SCFA production (Cho et al., 2012). The researchers in that study used a mix of different antibiotics and exposed the antibiotics to the dams rather than the neonates. Other studies also showed reduction of SCFA after antibiotic treatment (Mellon et al., 2000; Garner et al., 2009). Since we orally treated neonatal piglets after birth rather than indirectly via the dam, we expect that our model more accurately reflects what would be observed in infants exposed to amoxicillin.

In conclusion, by using a swine model for infants, we provide direct evidence for the positive correlation between early life antibiotic exposure and alterations in pancreatic development and function as well as impaired glucose metabolism later in life, suggesting the necessity of limiting antibiotic exposure and using more effective narrow-spectrum with fewer off-target harmful effects and alleviating the effects of antibiotics to the healthy gut microbiota. A recent study demonstrated the protective function of autoinducer-2 (AI-2), a member of a family of signaling molecules used in

quorum sensing, in promoting gut microbial balance by increasing microbial communication. Manipulation of AI-2 in antibiotic-treated individuals led to a significant increase in the phylum that was almost cleared after the antibiotic treatment (Thompson et al., 2015). Deeper investigations of the metabolic pathways for microbial-microbial communication and microbial-host interactions will help us prevent mitigate and even treat the problems caused by antibiotic use when antibiotics are required during severe infections.

Tables

Table 2.1 Duration for pancreatic islets digestion

Age of Donor Pancreas (PND)	First Digestion (min)	Second Digestion (min)
0 (week 0)	30	15
21 (week 3)	35	20
49 (week 7)	35	20

Table 2.2 Porcine-specific primer sequences used in quantitative RT-PCR analysis

Gene	Accession No.	Primer	Primer sequence (5'→ 3')	Ta (°C)	Amplicon length	Reference
IGF2	NM_213883.2	Forward	AGGGCATCCAAACCACAAAC	60	96 bp	Gondret et al., 2013
		Reverse	GGGTTCAATTTTTGGTATGTAACTTG			
INS	NM_001109772.1	Forward	CAGAAGCGTGGCATCGTG	60	76 bp	This study
		Reverse	CCTAGTTGCAGTAGTTCTCCAG			
PDX-1	NM_001141984.1	Forward	AAGTCTACCAAGGCTCACGC	60	159 bp	Liu et al., 2014
		Reverse	GCGCGGCCTAGAGATGTATT			
GPR41 (FFAR3)	XM_005664489.1	Forward	TGGAGACCTTACGTGTTG	57	75 bp	Haenen et al., 2013
		Reverse	CGAGGATGAGAAGTAGTAGAT			
GPR43 (FFAR2)	NM_001278758.1	Forward	CGTGTTCATCGTTCAGTA	57	76 bp	Haenen et al., 2013
		Reverse	GAAGTTCTCATAGCAGGTA			
MCT1(SLC16A1)	NM_001128445.1	Forward	GGTGGAGGTCCTATCAGCAG	60	169 bp	Metzler-Zebeli et al., 2012
		Reverse	TGAAGGCAAGCCCAAGAC			
MCT4(SLC16A3)	XM_003357925.2	Forward	CATCACGGGCTTCTCCTACG	60	163 bp	This study
		Reverse	GCGATTCACACACACACTGC			
IAP	XM_003133729.2	Forward	CTAAAGGGGCAGATGAATGG	60	105 bp	Arnal et al., 2014
		Reverse	CACCTGTCTGTCCACGTTGT			
APN	NM_214277.1	Forward	CAATATGCCGCCCAAAGGTTC	60	163 bp	Willing et al., 2009
		Reverse	CCGGATCAGGACGCCATTT			
OCLN	NM_001163647.2	Forward	ATCAACAAAGGCAACTCT	57	157 bp	Zhang et al., 2009
		Reverse	GCAGCAGCCATGTACTCT			
CLDN1	NM_001244539.1	Forward	TGATGAGGTGCAGAAGATGC	60	174 bp	This study
		Reverse	CCAGTGAAGAGAGCCTGACC			
MUC2	AK231524.1	Forward	CTGCTCCGGGTCCTGTGGGA	60	101 bp	Pieper et al., 2012
		Reverse	CCCGCTGGCTGGTGCGATAC			
ZO-1	XM_005659811.1	Forward	GAGTTTGATAGTGGCGTT	59	298 bp	Zhang et al., 2009
		Reverse	GTGGGAGGATGCTGTTGT			
GAPDH	NM_001206359.1	Forward	GTTTGTGATGGGCGTGAAC	60	147 bp	Willing et al., 2009
		Reverse	ATGGACCGTGGTCATGAGT			

Ta: annealing temperature

Figures

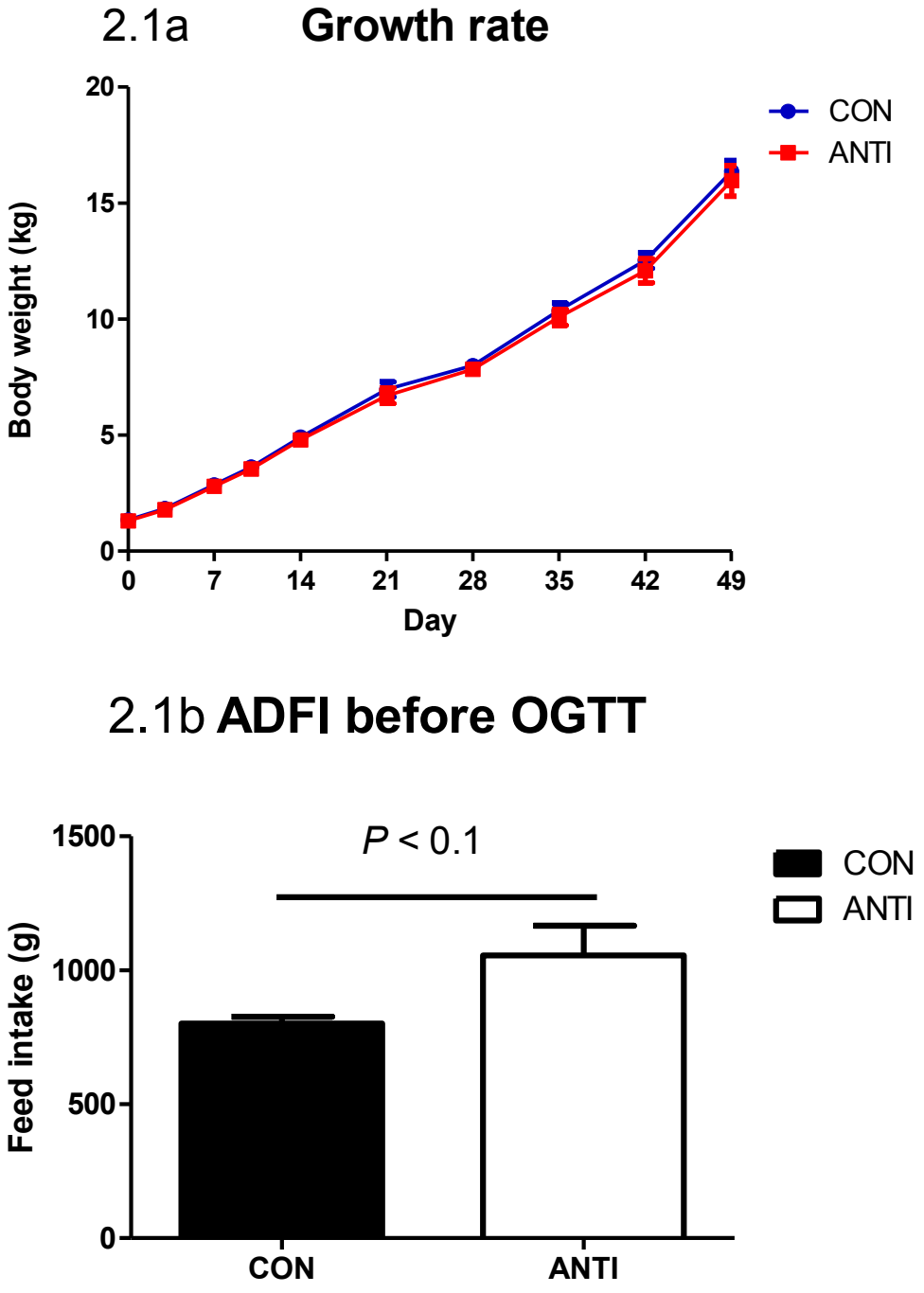

Figure 2.1 Effect of postnatal antibiotics on growth performance. Weight gain did not differ between ANTI and CON pigs (N = 16, 14) ($P > 0.05$). Pigs treated with antibiotics tended to have higher ADFI than CON pigs ($P < 0.1$).Data are presented as mean ± SEM.

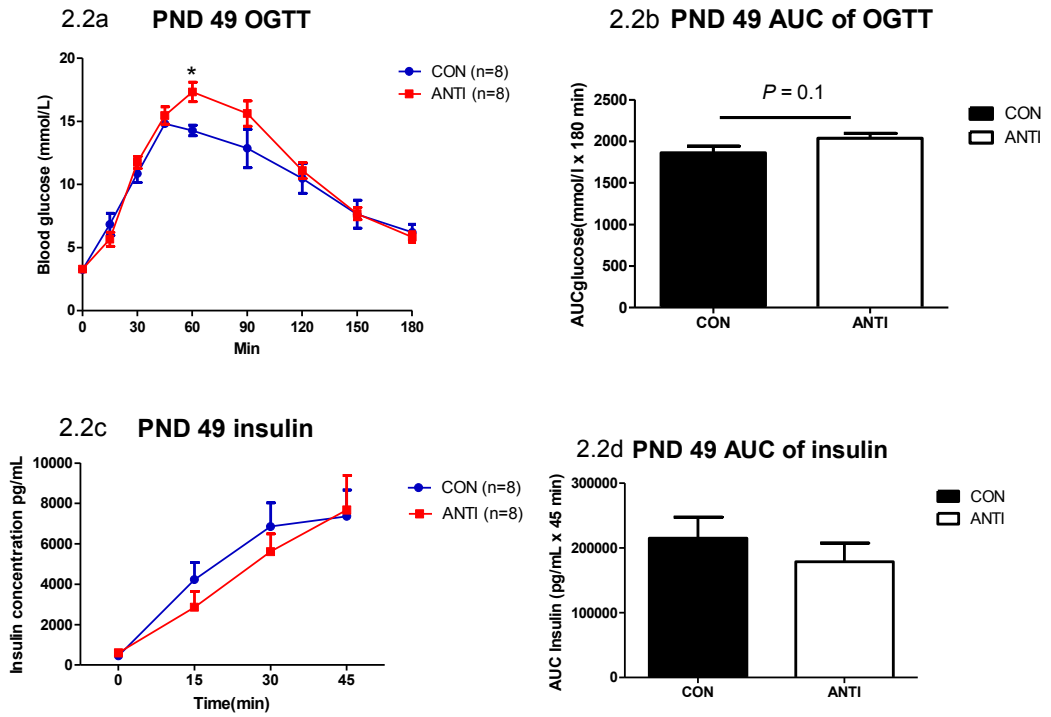

Figure 2.2 Early life antibiotic treatment alters response to glucose challenge later in life. a) At PND 49, glucose concentration of pigs from ANTI (N = 8) peaked significantly higher than CON (N = 8) ($P < 0.05$). b) Area under the curve (AUC) of OGTT was higher in ANTI but not significantly different from CON ($P = 0.1$). c) Insulin concentration and d) AUC of insulin for the first 45 min during OGTT were not significantly different between two groups. Data are presented as mean ± SEM. *$P < 0.05$.

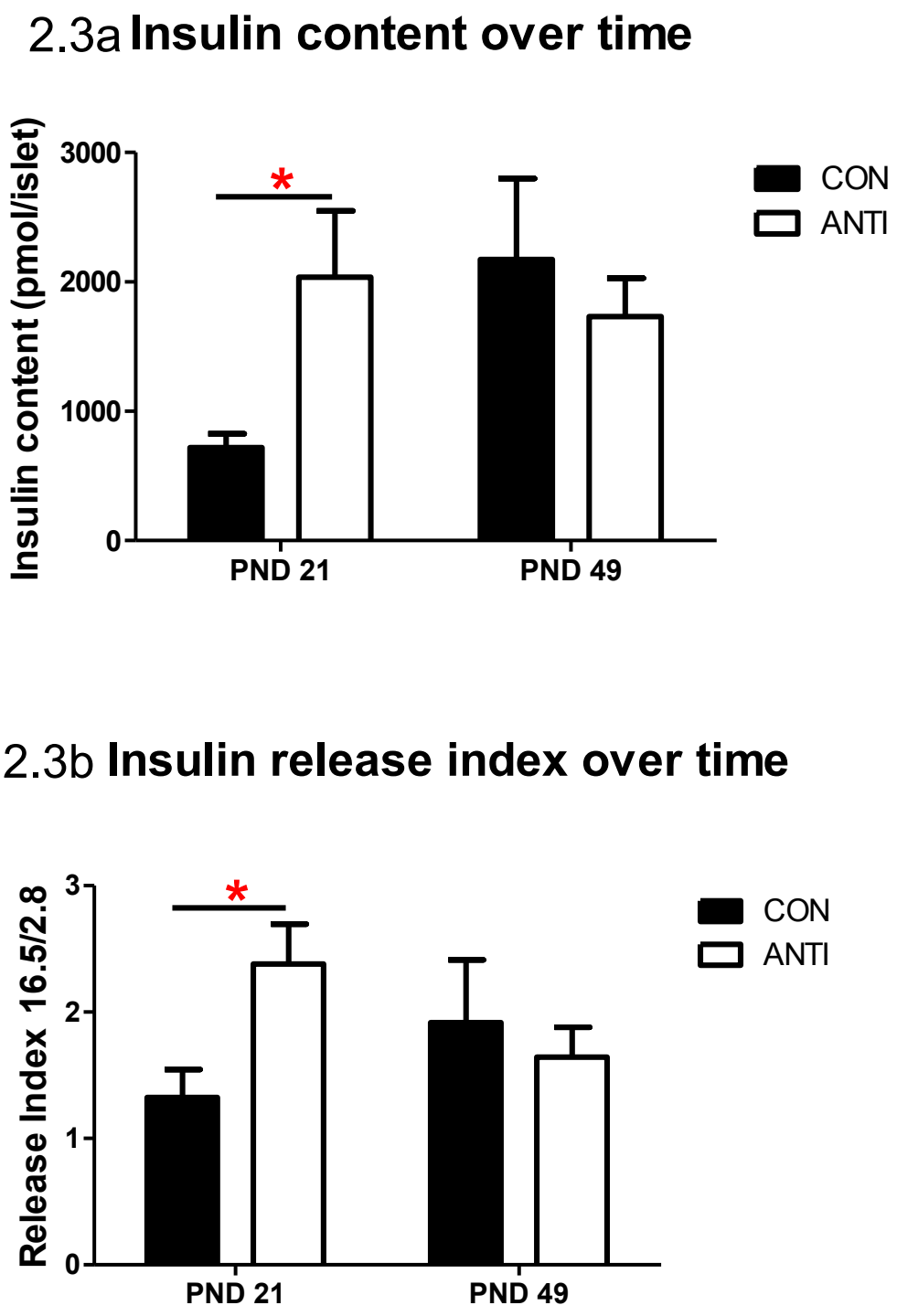

Figure 2.3 Glucose-stimulated insulin secretion assay (GSIS) in isolated pancreatic islets. At PND 21, islets from antibiotic treated pigs (N = 7) had higher insulin content than CON (N = 7) and ($P < 0.05$) released more insulin when challenged with glucose ($P < 0.05$). However, at PND 49 there was no longer a difference in the insulin content and release index. Data are presented as mean ± SEM. *$P < 0.05$.

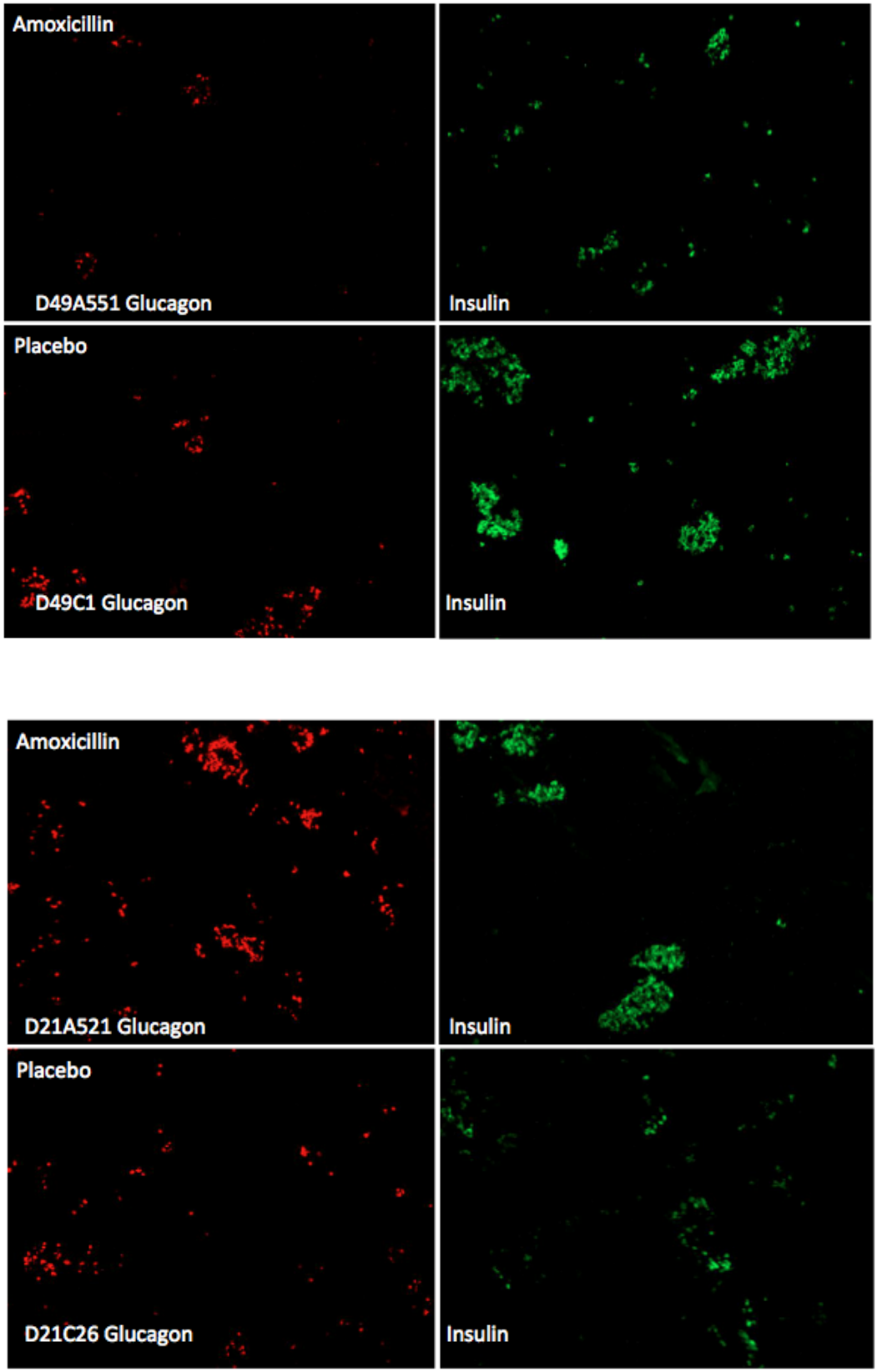

Figure 2.4 Immunofluorescence for insulin in fixed pancreatic tissue collected at PND 21 and 49. At PND 21, there was more insulin and glucagon in pancreatic tissues from ANTI. At PND 49, insulin in CON became higher than in ANTI.

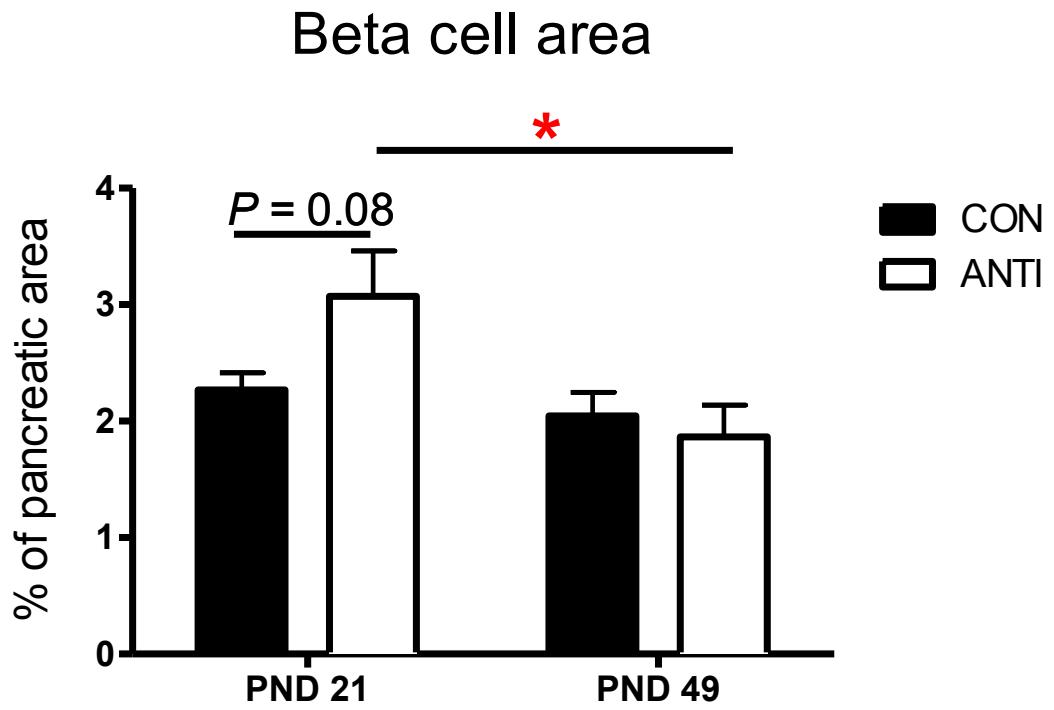

Figure 2.5 Early exposure to antibiotic alters pancreatic beta cell development. At PND 21, there was more insulin producing beta cells in pigs exposed to antibiotics than CON group ($P = 0.08$). At PND 49, the beta cell area decreased significantly in ANTI group compared with PND 21 ($P < 0.05$). Data are presented as mean ± SEM. *$P < 0.05$.

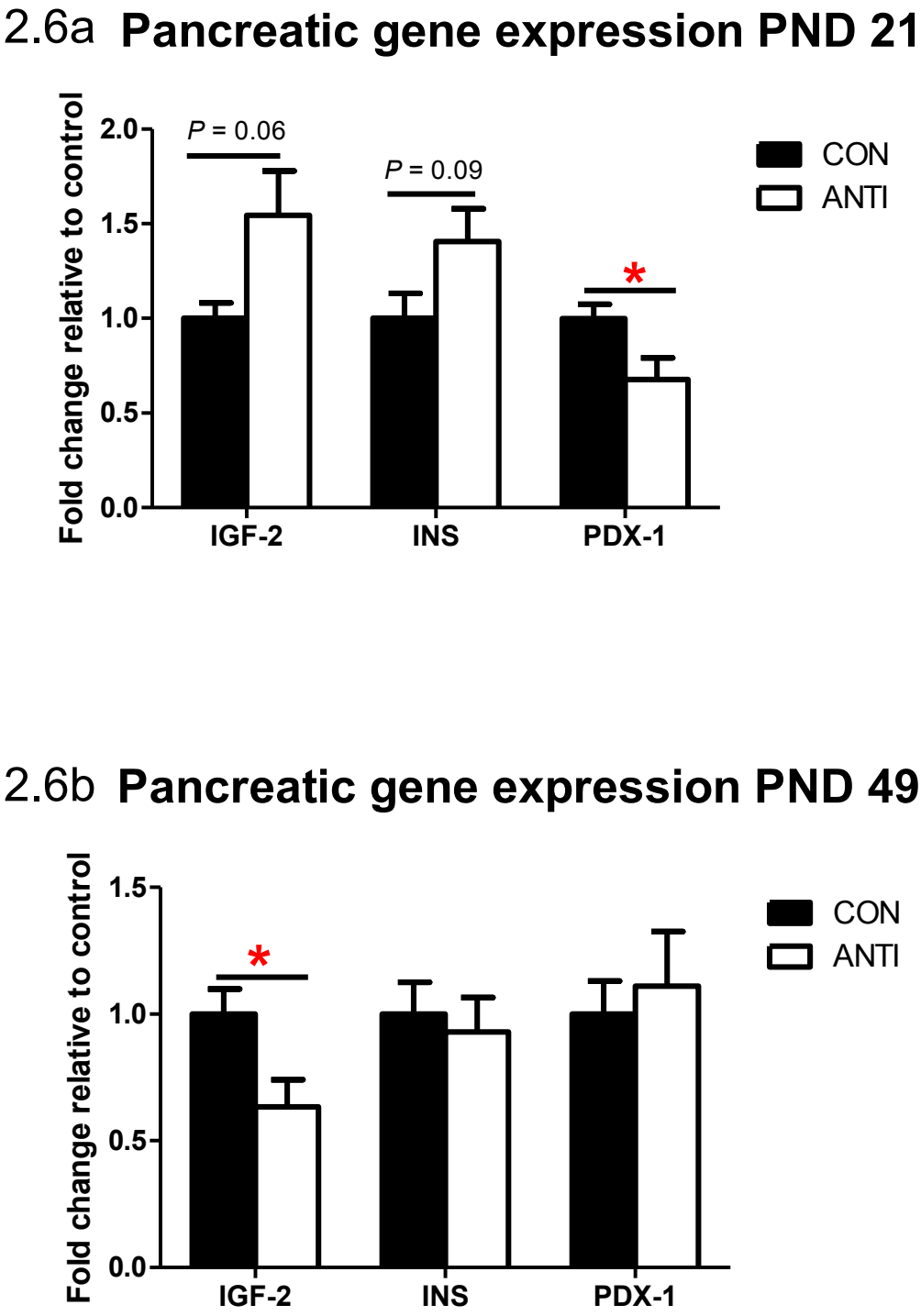

Figure 2.6 Early exposure to antibiotic results in differential expression of genes related to pancreatic islet development. At a) PND 21, expression of *PDX-1* was significantly decreased in ANTI ($P < 0.05$) while *IGF-2* and *INS* tend to express highly in ANTI ($P < 0.1$, N = 7). At b) PND 49, the difference between two groups became significant in *IGF-2* expression. Data are presented as mean ± SEM. *$P < 0.05$.

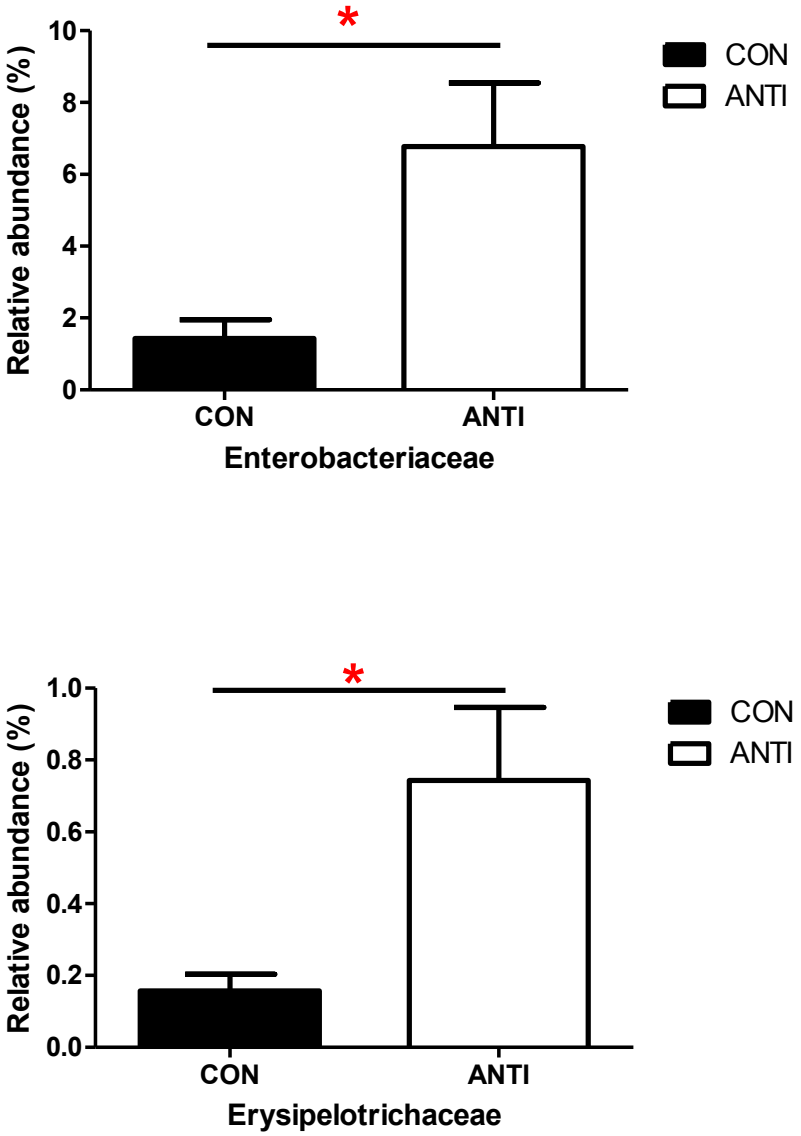

Figure 2.7 Amoxicillin enriched the percentage of Enterobacteriaceae and Erysipelotrichaceae of total bacteria. Microbial population was measured in amoxicillin treated (ANTI) and control (CON) pigs at PND 7. Enterobacteriaceae and Erysipelotrichaceae exhibited significantly higher abundance in ANTI (N = 12) than in CON (N = 11). Data are presented as mean ± SEM. *$P < 0.05$.

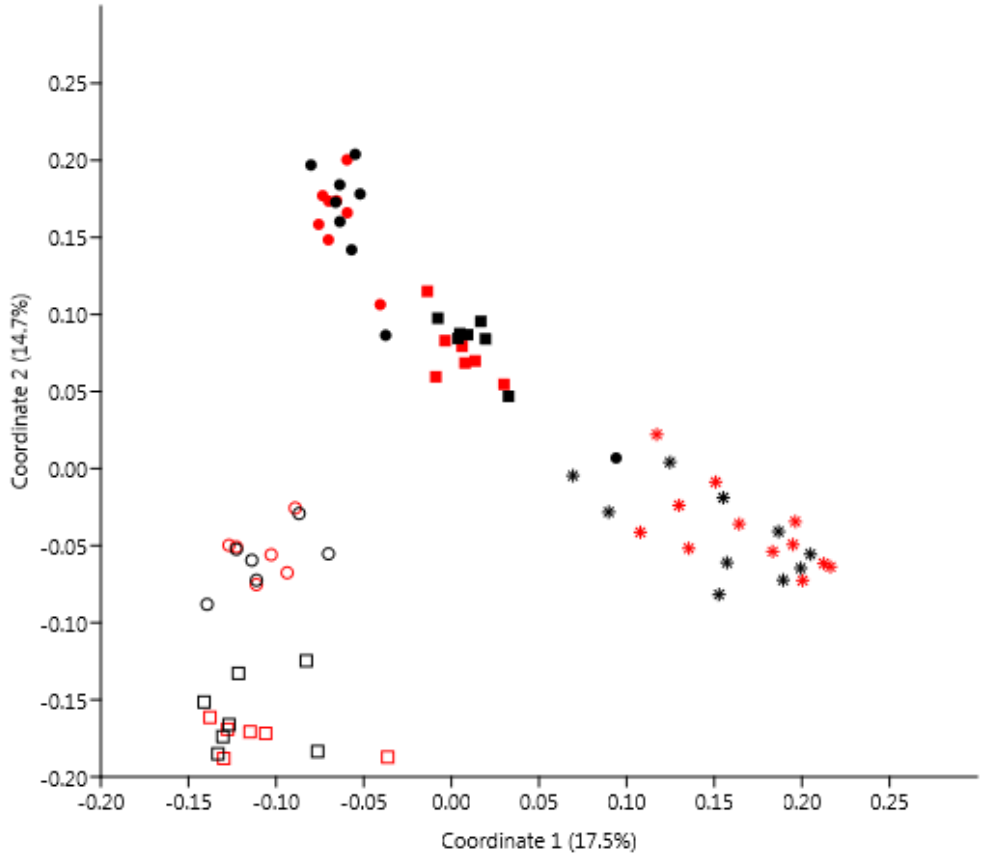

Figure 2.8 PCoA plot of community composition in antibiotic and placebo treated pigs. Microbial population of amoxicillin treated (ANTI) and control (CON) pigs were plotted at PND 7. Fecal samples and contents from ileum and distal colon were plotted as follow: ANTI PND 7 (Red star), CON PND 7 (Black star), PND 21 ileum (Hollow Square), PND 49 ileum (Hollow circle), PND 21 distal colon (Full Square) and PND 49 distal colon (Full circle). In the overall sample, datapoints did not cluster on a principle coordinate analysis (PCoA) scatter-plot as a function of antibiotic exposure. Datapoints were clustered as effects of sampling location and sampling time ($P < 0.001$).

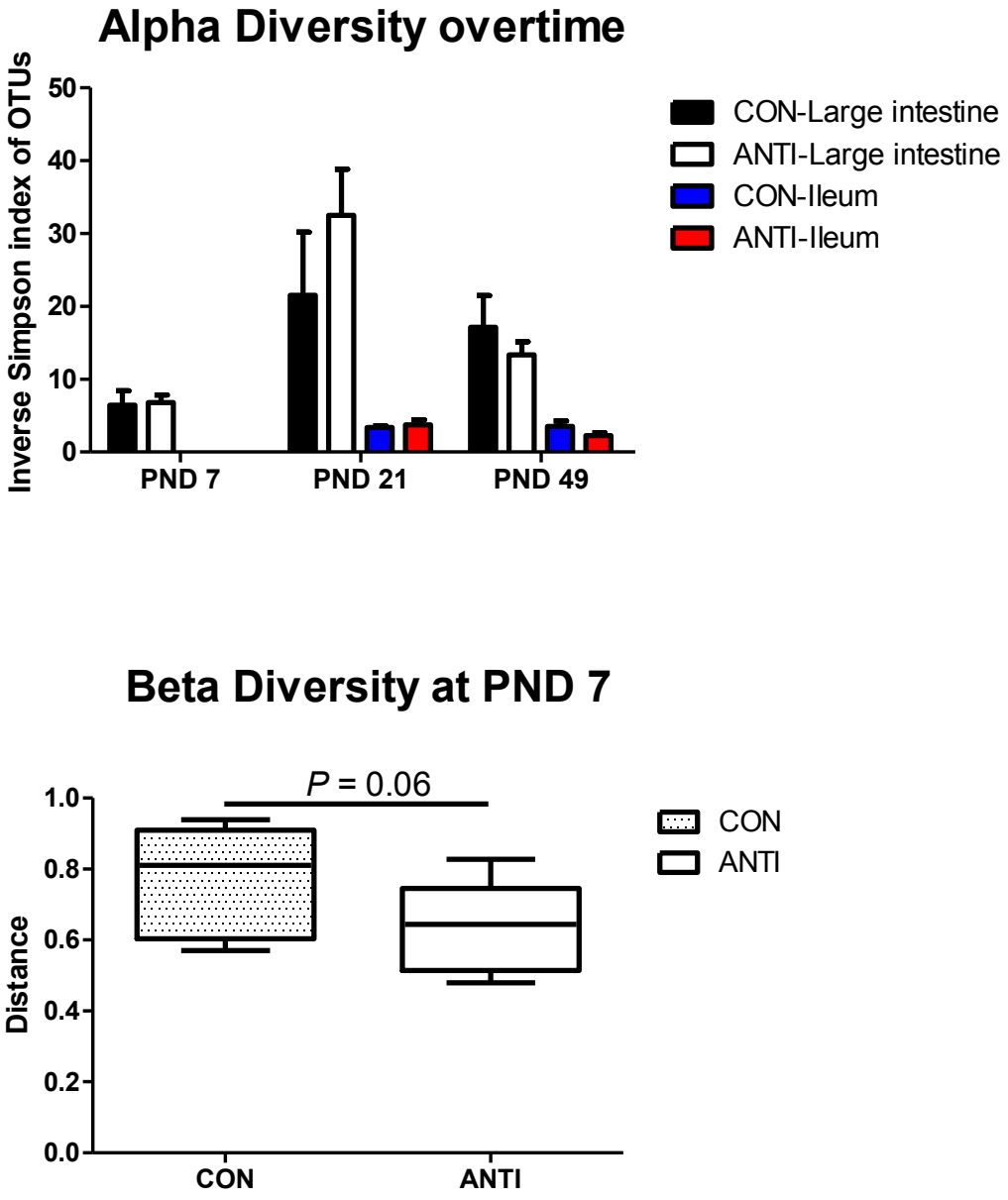

Figure 2.9 Alpha and beta diversity comparisons between amoxicillin and placebo treated pigs. Overall, there was no effect of antibiotic treatment on alpha diversity in gastrointestinal tract as indicated by the inverse Simpson-diversity index. However, there was a trend for reduced beta diversity in antibiotic treated pigs at PND 7 (P = 0.06). Data are presented as mean ± SEM.

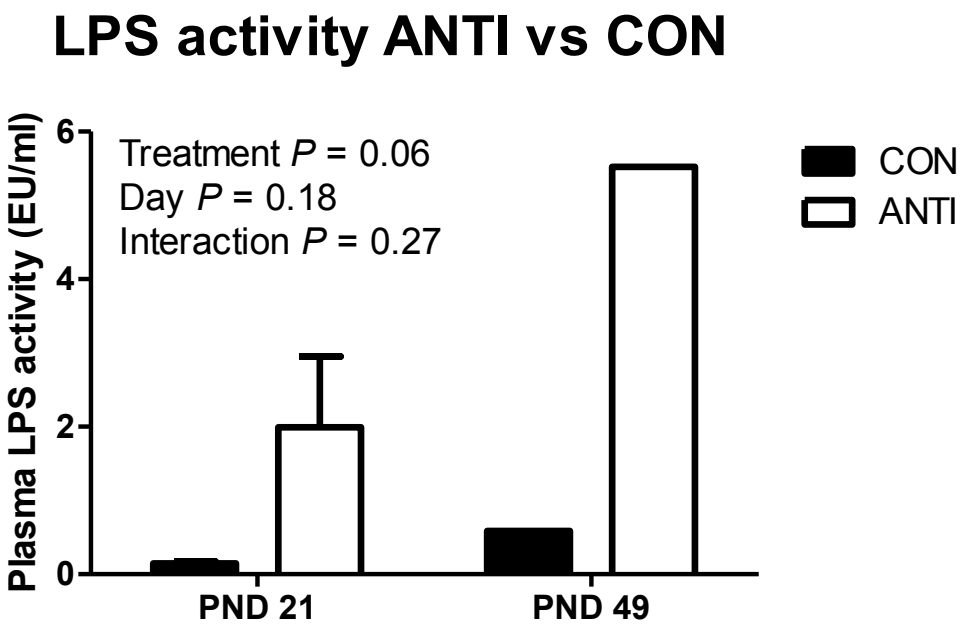

Figure 2.10 Effects of antibiotic on circulating LPS levels. There was a trend for higher plasma LPS activity for amoxicillin treatment ($P = 0.06$) by two-way ANOVA analysis. Data are presented as mean ± SEM.

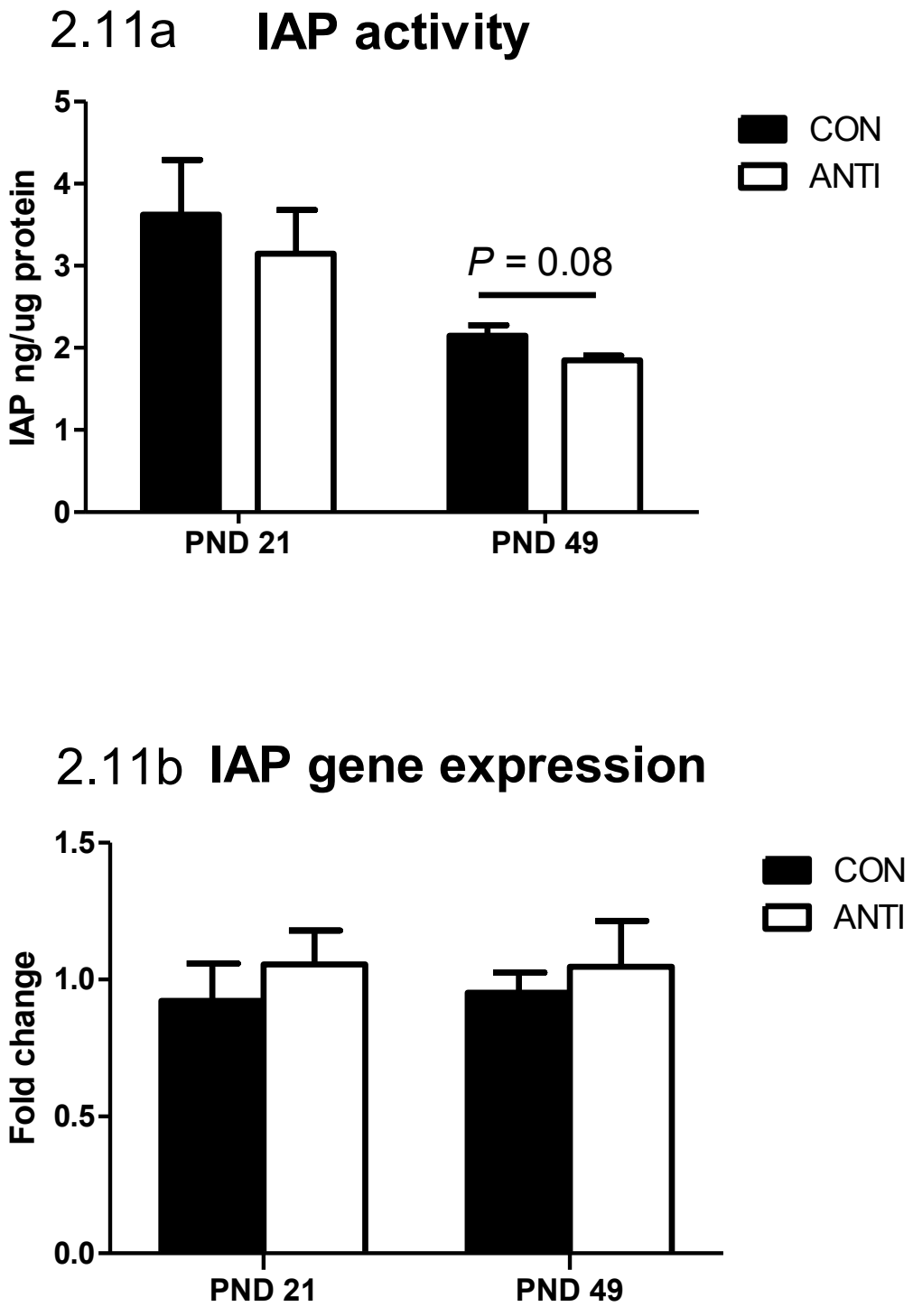

Figure 2.11 Intestinal alkaline phosphatase activity and gene expression levels in ileum. a) At PND 21 and PND 49, there was no significant difference between two treatments. However, ANTI group tended to have lower IAP amount at PND 49 in ileal mucosa (P = 0.08). b) There was no significant difference in two groups regarding the IAP gene expression in ileum whole tissue. Data are presented as mean ± SEM.

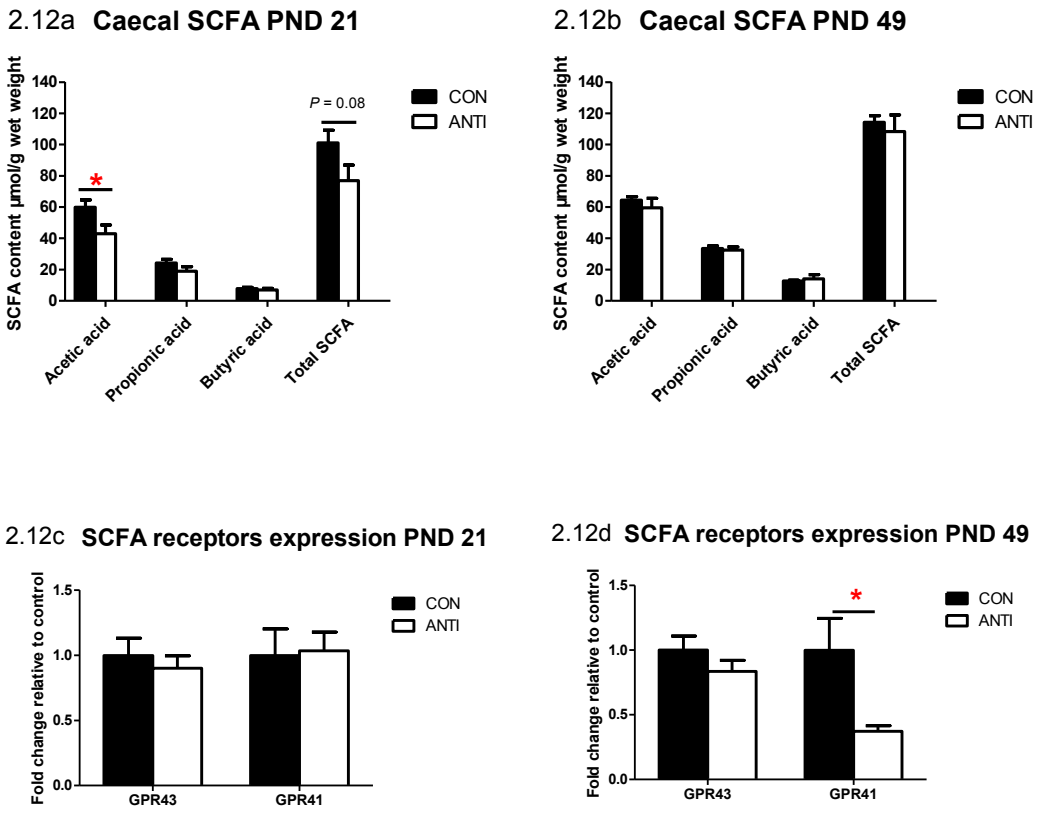

Figure 2.12 Amoxicillin alters caecal SCFA metabolism. Acetic acid was significantly lower in ANTI (N = 6) than in CON (N = 7) at a) PND 21. Total SCFA also showed the same trend ($P = 0.1$). However, no difference between postnatal amoxicillin and control pigs was seen at b) PND 49. Gene expression levels of two major SCFA receptors were shown at c) PND 21 and d) PND 49. There was no significant difference in two groups in *GPR43* expression at PND 21 and PND 49. At PND 49, ANTI group pigs showed significantly lower *GPR41* expression ($P < 0.05$). Data are presented as mean ± SEM. *$P < 0.05$.

- Chapter 3: HFD-induced Swine Model for the Study of Metabolic Syndrome

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3.0 Introduction

The incidence of metabolic syndrome has reached epidemic levels in recent years as a consequence of complex interactions between genetic, lifestyle, and environmental factors. Metabolic syndrome is characterized by the presence of the following symptoms: central obesity, glucose intolerance, insulin resistance, dyslipidemia, low-grade systemic inflammation and hypertension, which predispose to the onset of diabetes and cardiovascular diseases (Grundy et al., 2004). One of the major risk factors for the development of metabolic disorder is obesity, which is not a disease itself, but the condition often causes endocrine and metabolic changes and affects approximately 2.1 billion individuals worldwide (Ng et al., 2013). According to a recent scientific report of dietary guidelines published this year, 65 percent of adult females and 70 percent of adult males in the U.S. are overweight or obese and the current rates have persisted for more than 25 years (Dietary Guidelines Advisory Committee, 2015). Obesity results from an imbalance of energy intake and expenditure to regulate body weight and fat storage. Although genetic factors are

[3] Author Contribution: Conception and design of the experiments: Benjamin Willing and Jiaying Li. Performance of the experiments: Jiaying Li, Tingting Ju and Yanhua Gao. Analysis of the data: Jiaying Li, Benjamin Willing and Catherine Chan. Contribution of reagents/material/analysis tools: Jiaying Li and Catherine Chan. Diet formulation: Ben Willing, Catherine Field and Jiaying Li. Writing of the paper: Jiaying Li and Benjamin Willing

important for obesity development, sedentary lifestyle and a fat-enriched diet in developed countries have become a problem of upmost importance. Despite tremendous efforts dedicated to understanding obesity and decreasing the incidence, the mechanisms regulating the interplay between obesity, diabetes and β cell dysfunction in humans remain unclear. Human studies limit the mechanistic understanding, therefore, a large number of animal models ranging from fruit flies to non-human primates have been generated to study diet-induced obesity (Lai et al., 2014).

Choosing appropriate animal models is essential in replicating the results in human studies and in reducing the number of total animals used for research purpose. Among all the animal models, rodents are the most widely used due to their low cost, readily available gene knockout strains and easy management. However, they often fail to provide an accurate representation of human metabolic syndrome due to the marked differences in eating habit, metabolism, pancreatic and adipose tissue biology; complicating the translation of biomedical research findings to effective preventative or intervention therapies (Lai et al., 2014). Therefore, seeking alternative and complementary animals that are phylogenetically closer to humans is of considerable interest.

Humans and pigs share similar gastrointestinal structure and function, lack brown adipose tissue postnatally, and have pancreas morphology and overall metabolic features (Larsen et al., 2004). Importantly, the pig genome has been sequenced in recent years, facilitating the identification of putative disease causing variants and genomic characters of pigs (reviewed by Koopmans et al., 2015). Currently, there are several pig models being extensively used for investigation of metabolic syndrome, including Gottingen, Yucatan and Ossabaw (Bellinger et al., 2006). Among these, a large amount of animal models for obesity and diabetes are induced by administration of streptozotocin, which destroys pancreatic endogenous cells, reducing insulin

secretion substantially (Lenzen et al., 2008). Pigs have a large pancreatic β cell population, which makes them resistant to a diabetogenic environment. Since overt hyperglycemia usually doesn't develop within 6 months after HFD intervention, streptozotocin has been used to speed up the development of diabetes and is often combined with HFD feeding to promote metabolic syndrome (Koopmans et al., 2015). However, the dosage and the number of streptozotocin injections vary among studies considerably and increases complexity in understanding the process of metabolic disorder development and limit the models ability to accurately mimic human obesity and diabetes (Srinivasan et al., 2007). Furthermore, human T2D is not caused by a sudden loss of the pancreas. Instead, it is due to the progressive loss of β-cell function. Therefore, a diet-induced model is more suitable to study the prediabetic conditions caused by non-genetic factors in humans. In addition, naturally genetically leptin deficient pigs were also used for obesity studies, which develop obesity easily due to lack of leptin and hyperphagia (Torres-Rovira et al., 2012). However, most of people are not genetically leptin deficient, making translation of findings more challenging.

Obesity is characterized with a chronic systemic low-grade inflammation in most obese patients, which subsequently lead to insulin resistance and glucose intolerance (Bastard et al., 2006). Studies in recent years have demonstrated that a high-fat diet results in a shift in the gut microbiota, inducing low-grade endotoxemia, which has been indicated as an initiator for obesity and diabetes (Erridge et al., 2007; Cani et al., 2008; Zhao et al., 2013). Lipopolysaccharide (LPS) is a major component of the outer membrane in Gram-negative bacteria and was reported to induce insulinemia and obesity by 4 weeks of subcutaneous infusion (Cani et al., 2007). When facing higher LPS challenge, there are host defense responses to the alterations that help protect against inflammation. An important component of the defense system is the secretion of brush border enzyme intestinal alkaline phosphatase (IAP). IAP is a gut microbiota-regulating factor that suppresses *E.coli* growth and detoxifies bacterial LPS so as to limit inflammation (Campbell et al., 2010; Kaliannan et al., 2015). The

mechanisms of how high fat diet induces gut microbial changes, the subsequent LPS activity alterations and how the host responds to the changes remain to be explored.

The aim of this study was to evaluate the young crossbred (Duroc × Large White/Landrace) pig as a model of childhood obesity in the metabolic response to a dietary challenge. The pig model used in this study effectively recapitulated the pathophysiology in a manner similar to metabolic syndrome development in humans.

3.1 Materials and Methods

3.1.1 Ethics Statement

This study was performed according to the guidelines provided by Canadian Council on Animal Care and with approval of the University of Alberta Animal Care and Use Committee (AUP00000922). Pigs used in this study were managed according to approved protocols at the Swine Research and Technology Centre (SRTC), University of Alberta.

3.1.2 Animals and Experimental Design

One litter of crossbred (Duroc × Large White/Landrace) piglets (1.39 ± 0.07 kg) born to 2nd parity sow, were randomly assigned as individual pigs to high fat diet (HFD) and standard low fat diet (LFD) treatments. Pigs were weaned at 21 d of age and moved to an assigned nursery room, penned by litter with unlimited access to feed and water. Nursery pigs were fed with a phase-feeding programme according to SRTC nursery guidelines. Pigs were moved to metabolic pens two weeks before oral glucose tolerance test (OGTT) and housed individually in fully slatted pens (1.2 m × 0.9 m). The pigs were provided with *ad libitum* access to feed and water from a nipple-in-bowl drinker (BALP, Charleville-Mezieres, Cedex, France). All animals

were housed at a temperature of 22°C to 25°C with a 12-hour dark: light cycle. Pigs were vaccinated as per standard herd protocol, with circovirus at weaning, erysipelas and Ileitis at week 7 before OGTT. OGTT was conducted at 7 and 11 weeks of age. After the first OGTT, six of the pigs were fed HFD and the other six were fed with LFD diet at libitum for 5 weeks until the end of the trial. At the beginning of the differential feeding, there was no difference in body weight in LFD (15.35 ± 0.72 kg, N=6) and HFD (15.28 ± 0.44 kg, N=6) groups. The diets (Table 3.1) were formulated to meet or exceed the nutrient requirements of pregrower pigs. The LFD is a control diet purchased from HI-PRO Feeds (Sherwood Park, AB) to meet the nutrient requirements for swine. The HFD consisted of high percentage of saturated fatty acids in the diet formulated by this lab with 44 percent of energy provided by fat. To avoid any deficiencies, essential amino acids, essential fatty acids, vitamins, and minerals were formulated to match metabolizable energy. Body weight data was taken every week. Individual body weight and feed disappearance were used for calculation of average daily gain (ADG) and average daily feed intake (ADFI).

3.1.3 Blood Glucose and Plasma Insulin Measurement

Oral glucose tolerance tests (OGTT) were performed after overnight fasting, essentially as described (Montelius et al., 2014) with some modifications. Briefly, before OGTT, pigs were allowed an acclimatization period of 2 weeks during which they were trained in the experimental procedures including being picked up by handlers and adapting to staying in a sling. An hour before OGTT, pigs ears were cleaned and Lidocaine/prilocaine cream (EMLA Cream; AstraZeneca, Mississauga, Ontario) was applied to the skin overlying the marginal veins of the pig ears to reduce pain throughout OGTT. During OGTT, glucose was measured in whole blood with a glucometer by ear pricking (Accu-Check Compact Plus; Roche Diagnostics) over a 3 h period. Fasting plasma glucose (FPG) concentration was measured before OGTT. Animals were then offered a meal consisting of 50g of ground pregrower fodder

mixed with 2g/kg glucose solution. The meal was eaten from a bowl under supervision. After glucose detection, additional 150µl blood samples were collected into Eppendorf tubes by 70µl microhematocrit capillary tubes coated with ammonium heparin (Fisher Scientific) every 15 min from -15 to 60 min, then every 30 min to 180 min for insulin measurement. Samples were centrifuged at 8,000×g for 10 min at 4°C and supernatant were collected and stored at -80°C until assayed for insulin by ELISA, according to the manufacturer's instructions (Alpco Diagnostics, Salem, N.H., USA). Area under the curve were calculated in accordance with established methods (Wolever, 2004)

3.1.4 Animal Euthanasia and Sampling

At PND 84, pigs from both groups were euthanized for sample collection. General anesthesia was induced in pigs with an injection of 16mg/kg ketamine HCl (Ketalean; Bimeda-MTC, Cambridge, ON, Canada), 2.2 mg/kg xylazine (Rompun; Bayer Inc., Toronto, ON, Canada), and 6mg/kg azaperone (Stresnil; Janssen Pharmaceutica, Beerse, Belgium). Afterwards, 5% isoflurane (Isoflo, Abbott Laboratories Ltd., Saint-Laurent, Quebec, Canada) was provided using a face mask. Pigs were immediately exsanguinated. After exsanguination, the abdominal cavity of each pig was opened. The entire small intestines were subsequently removed. Ileum (15cm proximal to the ileo-caecal junction) was carefully dissected. Intestinal samples were opened longitudinally and rinsed with ice cold PBS. Digesta was removed and mucosal samples were collected from these tissues by scraping the mucosal lining with a glass slide. These samples were immediately snap-frozen in liquid nitrogen and then stored at -80°C for subsequent enzyme activity measurement. Back fat thickness was measured to indicate subcutaneous fatness of the pigs. A scalpel was used to make an incision on the back at the level of the 4th to last rib, and the area between the dermis and muscle layers were measured with a ruler.

3.1.5 Circulating Endotoxin Detection

Plasma endotoxin concentrations were measured by a kinetic fluorescent assay using the PYROGENT-5000 kit (Lonza, Mississauga, Canada). All procedures were performed under non-pyrogenic conditions. Briefly, the plasma samples were diluted 5 × in non-pyrogenic water and were heat inactivated at 70°C for 30 min. A 100 µl aliquot of the samples and standards were added to a 96 well round bottom plate and incubated at 37°C for 10 min. After incubation, 100µl of the reconstituted PYROGENT™-5000 Reagent assay buffer was added to the plate and a kinetic reading was taken up to 1h at 37°C. Thereafter, the relative fluorescence unit for each well was determined (absorbance 340nm). The concentration of the endotoxin was interpolated from the standard curve constructed from the standards and corrected for sample dilution.

3.1.6 IAP Activity Detection

Alkaline phosphatase activity in the ileal mucosa was measured by SensoLyte® pNPP Alkaline Phosphatase Assay Kit Colorimetric (AnaSpec, product #72146, USA). The assay was performed according to the manufacturer's protocol for biological samples. Specifically, approximately 40mg of frozen ileal mucosal sample was thawed in 1ml of ice-cold homogenizing buffer (provided by the kit) and homogenized for 1min with glass beads (1.0mm dia, BioSpec Products, Bartlesville, USA). The resulting homogenate samples were analyzed for protein content and enzyme activity kinetics for IAP. The samples were diluted 100 times for enzyme detection and 10 times for protein concentration measurement. IAP activity was normalized to protein concentrations of total homogenate, as measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Ottawa, ON, Canada). The intensity of color corresponding to the alkaline phosphatase activity was read using SpectraMax M3 Microplate Reader (SpectraMax M3, Sunnyvale, CA) and the absorbance was measured at 405 nm.

3.1.7 Statistical analysis

Data were expressed as mean ± SEM. Analysis of variance (ANOVA) was used to test for differences in glucose; insulin concentration in OGTT and body weight, adjusted by Bonferroni post hoc tests. Glucose and insulin data were converted to area under the curve (AUC) values by the trapezoidal method. Comparisons between the pigs on different diets were performed using independent-samples Student's t test to evaluate the associations of continuous variables after normality tests. An alpha value of $P \leq 0.05$ was considered significant and a P value between 0.1 and 0.05 was considered as a trend. Data were analyzed using GraphPad Prism v. 6.02 (La Jolla, CA) and IBM SPSS Statistics, version19.

3.2 Results

3.2.1 Effects of HFD on Growth Performance

After the first OGTT, pigs were fed differentially with HFD and LFD for 5 weeks. To examine whether HFD affected growth traits, feed intake, energy consumption, body weight, average daily gain and carcass back-fat thickness were compared (Figure 3.1, 3.2). Pigs on HFD reduced feed intake significantly to balance energy consumption ($P < 0.01$). However, even with lower feed intake, the metabolizable energy consumed was higher in HFD pigs ($P < 0.0001$). High fat rapidly increased average daily gain resulting in an average 5kg difference in body weight of pigs in HFD group over a period of 5 weeks ($P < 0.05$). The adiposity of these pigs was reflected by the back-fat depth with significantly higher back fat in high fat fed pigs ($P < 0.0001$). Thus, animals in HFD group showed obesity and particularly, adiposity after 5 weeks of differential feeding, a first symptom of metabolic syndrome.

3.2.2 HFD Feeding Alters Glucose Metabolism

Obesity is the central symptom of metabolic syndrome and is highly connected to diabetes. Several indicators are recognized as major components that predispose diabetes, including glucose intolerance, insulin resistance and pancreatic dysfunction. To examine the glucose metabolism in pigs fed with HFD, an OGTT was performed at PND 49 before HFD feeding. In a pilot study it was confirmed that blood glucose sampling by ear pricking caused minimal stress as indicated by stable blood glucose levels over time in the absence of glucose challenge. This test was used to assess how efficiently the pig was able to maintain blood glucose concentrations. It is usually used to test for diabetes, insulin resistance and impaired beta cell function. Before high fat diet was fed to pigs, there was no statistical difference in glucose concentration (P = 0.71) or area under the curve (AUC) (P = 0.87) of glucose concentration (Figure 3.3) and pigs responded similarly to high glucose challenge. Within 4 weeks, HFD feeding was shown to have significant effect ($P < 0.01$) on host glucose control during OGTT by two-way ANOVA analysis. HFD pigs tended to have higher fasting glucose levels than control group (P = 0.06). Area under the curve (AUC) of glucose concentration during OGTT also showed a trend (P = 0.09) toward elevated glucose levels, thus lower glucose tolerance, in the HFD group. Although, 3 hours after the glucose challenge, glucose levels of pigs from both groups did not return to the fasting values, pigs fed with HFD showed significantly higher glucose concentration at the last time point than control pigs ($P < 0.01$).

Significant lower insulin secretion during the first 60 min of OGTT was observed in HFD feeding group ($P < 0.05$). In addition, there was a trend of lower AUC of insulin in HFD pigs (P = 0.09). The OGTT showed that, in control animals, the plasma glucose levels started to increase after 15 minutes and reached a peak around 60 minutes and rapidly decreased to fasting values. Assessment of insulin levels in these pigs showed a well-characterized acute insulin response that decreased after the 30 min time point. Together, these data indicate impaired response to glucose challenge

after high fat feeding.

3.2.3 Changes of LPS and Host Responses to HFD Feeding

To determine whether HFD feeding altered circulating LPS concentration, we measured LPS in circulating blood at PND 84 (Figure 3.4). No significant difference in LPS activity between the high fat and low fat feeding was observed ($P = 0.44$). We also measured the capacity of the host to detoxify LPS by measuring IAP activity (Figure 3.5). There was no statistical difference in both groups at PND 84 ($P = 0.15$)., suggesting that the HFD did not increase LPS levels or start the host responses to the LPS challenge in five weeks of feeding.

3.3 Discussion

The first use of HFD in an animal obesity model dates back to the 1940s when rats were fed with an extreme HFD (70% energy from fat), which was much higher than the regular high fat diet consumed by human beings (Samuels et al., 1942; Ford et al., 2013). Over the decades, researchers have been using high fat diet with different compositions, which have made the data hard to translate to human condition. Given the broad range of dietary choices, there is no consensus on the ideal diet-induced obesity model. The typical American diet is about 35% of energy from fat, 15% from protein and 50% from carbohydrate (Last et al., 2006). Human studies have shown that high-fat diets with more than 30% of energy from fat can easily induce obesity (Jequier et al., 2002, Hill et al., 2000). In the present study, to avoid dilution of required micronutrients and macronutrients, rather than just simply add fat to a standard control feed we formulated a diet that is low in carbohydrate and high in fat. No symptoms of protein or micronutrients deficiency were observed during the experiment.

Consistent with several diet-induced obesity studies, animals on high-fat diet had lower voluntary feed intake (Pfluger et al., 2008; Liu et al., 2014) but much higher caloric consumption compared with the control group during high fat feeding (de La Serre et al., 2010; Barclay et al., 2013). This phenotype can be explained by the effects of fat or energy density on satiety. The mechanisms include the release of gut hormones and regulation of gastric emptying and intestinal transit (Westerterp-Plantenga et al., 2012). Although few studies have investigated the effects of different fatty acids on feed intake, based on the previous published papers, fat exerts much less satiety compared with carbohydrate and protein, suggesting that energy consumption is an important driver of feed intake (Montmayeur et al., 2009; Hariri et al., 2010).

The results in the present study indicate that crossbred production pigs can develop the prodrome of metabolic syndrome when these animals have free access to high fat diet for 4 weeks starting from 7 weeks of age. By the end of differential feeding, pigs on high fat diet had higher body weight and 86% more back-fat than LFD pigs, confirming our high-fat diet induces obesity and adiposity in this breed, although other phenotypes that characterize metabolic syndromes such as dyslipidemia and hypertension, should be examined in future studies. Furthermore, the effects of the diet on glucose metabolism were observed fairly early after starting the differential feeding. With no differences at the beginning in glucose levels and insulin secretion, 4 weeks of high fat diet led to significantly lower insulin release and glucose intolerance. It worth mentioning that high fat feeding does not always lead to consistent changes in glucose disposal or insulin secretion. Normoglycemia, slight hyperglycemia and the development of T2D have been reported due to different diet compositions (reviewed by Buettner et al., 2012). Gene expression of insulin secretion regulators in β-cells and pathophysiology of pancreatic islets should be examined to explore the underlying mechanisms in future studies.

It has been reported by several studies that HFD can alter microbial changes in the gut and disturb the normal fermentation and metabolism of resident microbes in the intestine, which is associated and causally linked to metabolic diseases in humans and mice (Daniel et al., 2014). An impaired gut barrier resulting in increased endotoxemia is hypothesized to be the main driver, which could further lead to insulin resistance caused by inflammation in pancreas and adipose tissue (Serino et al., 2012). In a Sprague-Dawley rat model for obesity, high fat diet led to increased plasma LPS, impaired tight junctions and decreased IAP activity in duodenal mucosa with 8 weeks of differential feeding (de La Serre et al., 2010). Notably, these symptoms were only observed in obesity prone rats, not in obesity resistant rats despite the same genetic background and the same high fat diet. However, no difference in plasma LPS activity was detected between high or low fat feeding in our study. This could be due to shorter high fat feeding in our study and the differences between species. The brush border enzyme IAP that detoxifies LPS did not show difference in ileal mucosa. This may be associated with differences in species and sampling site or that the pigs were not sufficiently challenged.

Using the pig as metabolic syndrome model has its own limitations. Obesity often occurs long in advance of diabetes. It can take several months to become diabetic in rodents. The natural progression towards T2D is difficult to mimic in pigs since pigs develop diabetes rarely (Buettner et al., 2007; Gerstein et al., 2006). Similarly, in mice, the rapid induction of obesity by HFD usually lacks the T2D associated complications, which often develop in years, mostly starting long before overt hyperglycemia. Phenotypes observed in this study are more similar to the progressive impaired metabolic regulation rather than fully established diabetic status. The crossbred pig raised may not be an ideal model for studying adult obesity due to their ultimate size (250 - 300kg), however, they make a good alternative obese model for HFD-induced child obesity and prediabetes. Further studies need to be done to explore the availability of using this model for other research, i.e. the effects of childhood high fat

diet on early life programming and metabolic syndrome development.

Tables

Table 3.1 Ingredients and chemical composition of the control and HFD (as fed-basis)

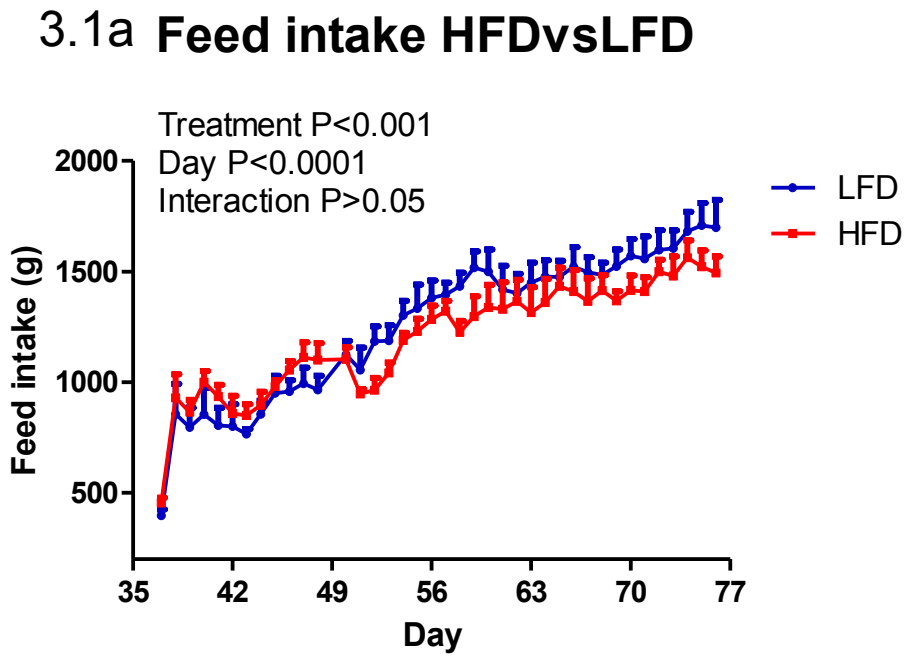
	Diet	
Ingredients	LFD[a] (g/kg)	HFD (g/kg)
Wheat, ground	599.7	379.5
Soybean meal, 460g CP/kg	123	330
Distillers wheat husky	50	0
Corn	94	0
Oat	59	0
Fish meal -Menhaden 620g CP/kg	0	60
Fat	25	188.1
Flax oil	0	1.9
Limestone / glass rock	15	8.6
Mono/dical phoshate	4.3	7
Salt	4.7	5
L-Lysine HCl 780g/kg	8.3	3.9
L-Threonine 990g/kg	3.3	2.3
DL-Methionine 990g/kg	2.7	1.4
L-Tryptophan 990g/kg	0.4	0.3
Vitamin mix[b]	0	6
Mineral mix[c]	0	6
Others	10.6	0
Digestible energy (kcal/kg)	3532.63	4314.51
Metabolizable energy (kcal/kg)	3336.87	4102.62
Net energy (kcal/kg)	2499.9	3097.95
Crude protein (%)	18.12	24.85
Crude fat (%)	4.28	21.03
Lysine (%)	1.30	1.70
Threonine (%)	0.87	1.12
Methionine (%)	0.48	0.54
Tryptophan (%)	0.24	0.34

a LFD is a standard feed pallet produced by HI-PRO FEEDS, Sherwood Park, AB.

b Supplied per kilogram of diet: 9000 IU of vitamin A, 9000 IU of vitamin D, 60 IU of vitamin E, 4.8 mg of menadione, 3 mg of thiamine, 6 mg of riboflavin, 1.8 mg of pyridoxine, 0.018 mg of vitamin B_{12}, 45 mg of niacin, 18 mg of pantothenic, 3 mg of folic acid and 0.3 mg of biotin.

c Supplied per kilogram of diet: 60 mg of Cu, 90 mg of Fe, 30 mg of Mn, 0.6 mg of I, 150 mg of Zn and 0.36 mg Se.

Figures



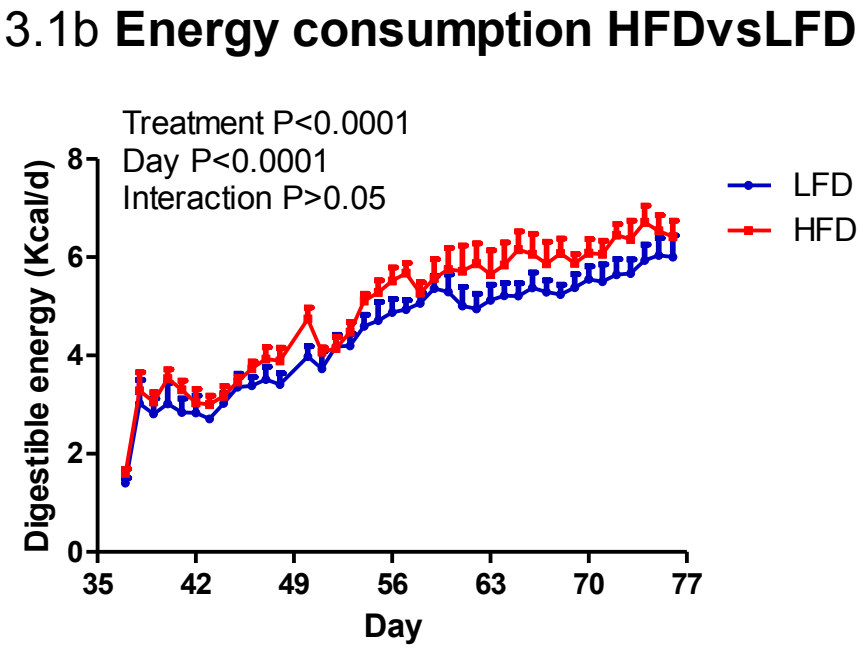
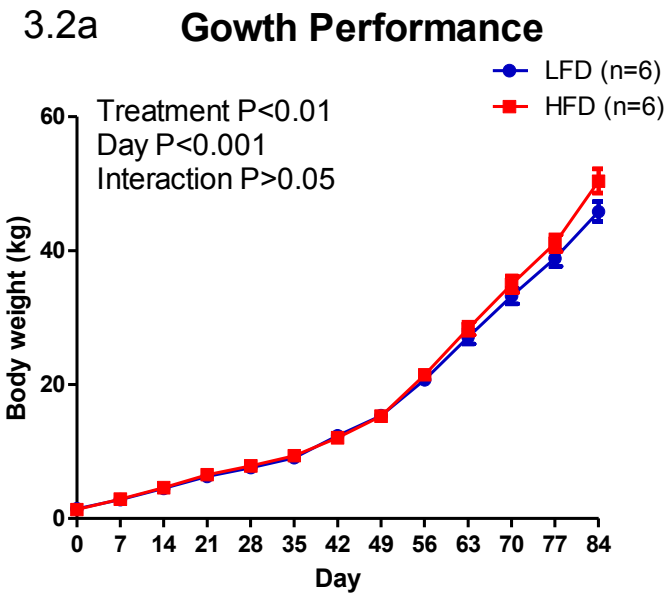
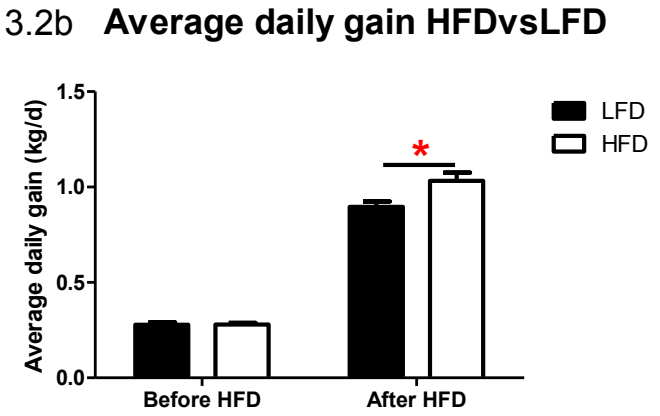


Figure 3.1 HFD-induced changes in feed intake (a) and energy consumption (b) over time after differential feeding in control pigs with LFD (blue line) and pigs fed on HFD (red line) with *ad libitum* access. Data are presented as mean ± SEM.





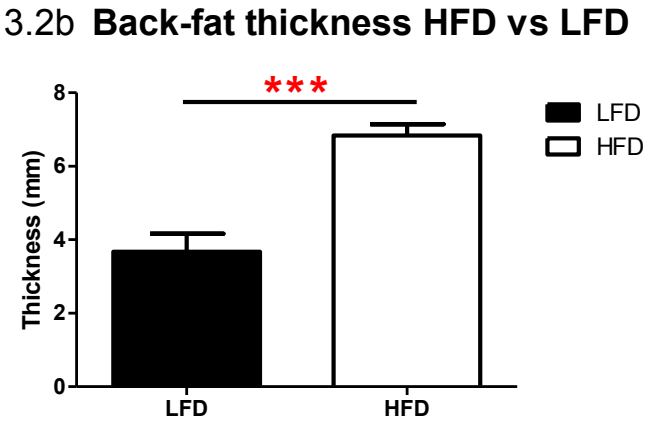


Figure 3.2 HFD effects live weight (a), average daily gain (b) and back-back depth (c) over time after differential feeding in control pigs with LFD (blue line or black bars) and pigs fed on HFD (red line or white bars) with *ad libitum* access. Data are presented as mean ± SEM. **P* < 0.05, ***P* < 0.01, *** *P* < 0.001.

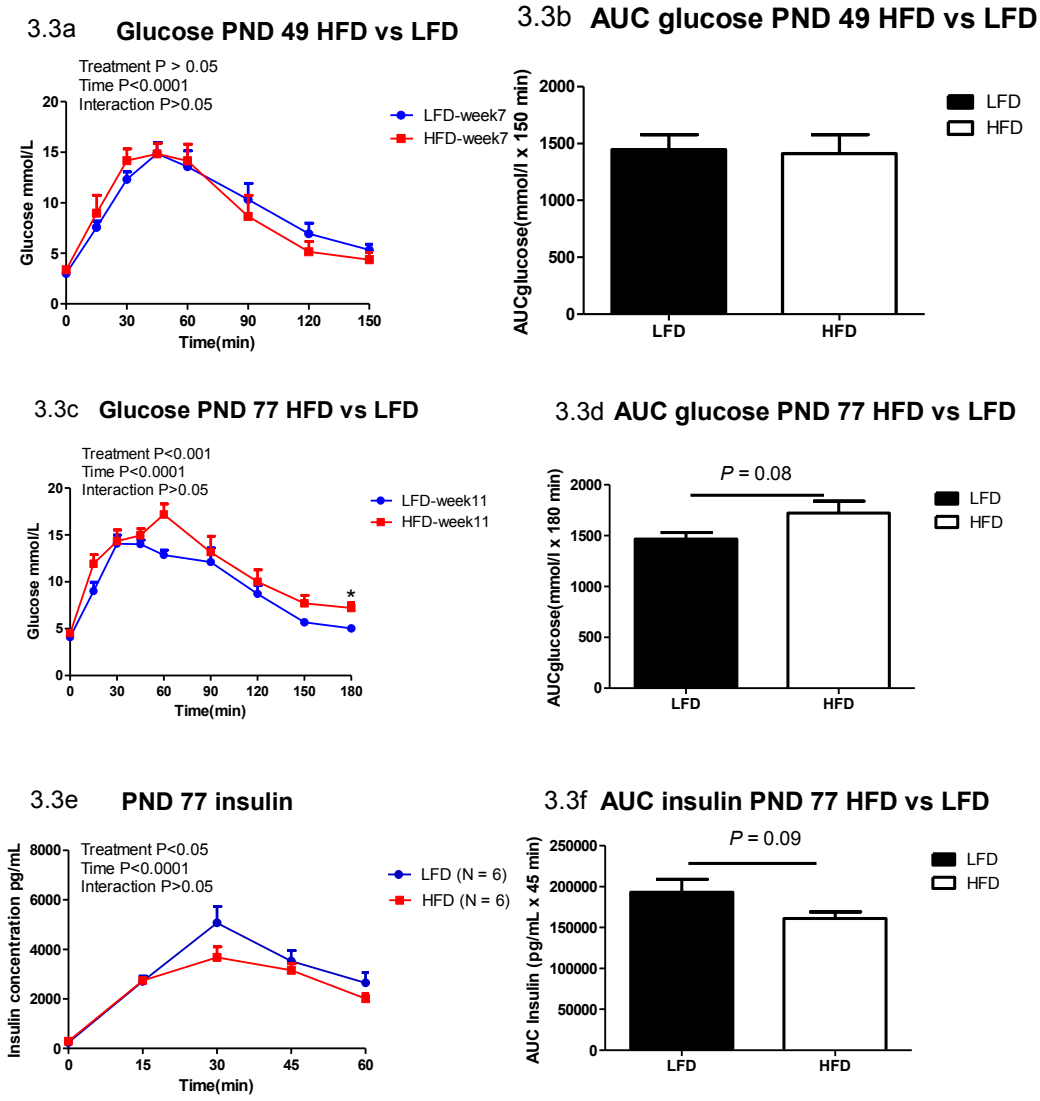


Figure 3.3 High fat diet feeding alters response to glucose challenge. No significant difference observed at PND 49 in a) glucose concentration or b) area under the curve of glucose concentration during the period. HFD treatment showed significant effect on c) glucose concentration ($P < 0.01$) and there was a trend for d) higher AUC in HFD feeding group ($P = 0.08$). Significant lower e) insulin ($P < 0.05$) and a trend of lower f) AUC ($P = 0.09$) were observed in HFD group. Data are presented as mean ± SEM. *$P < 0.05$.

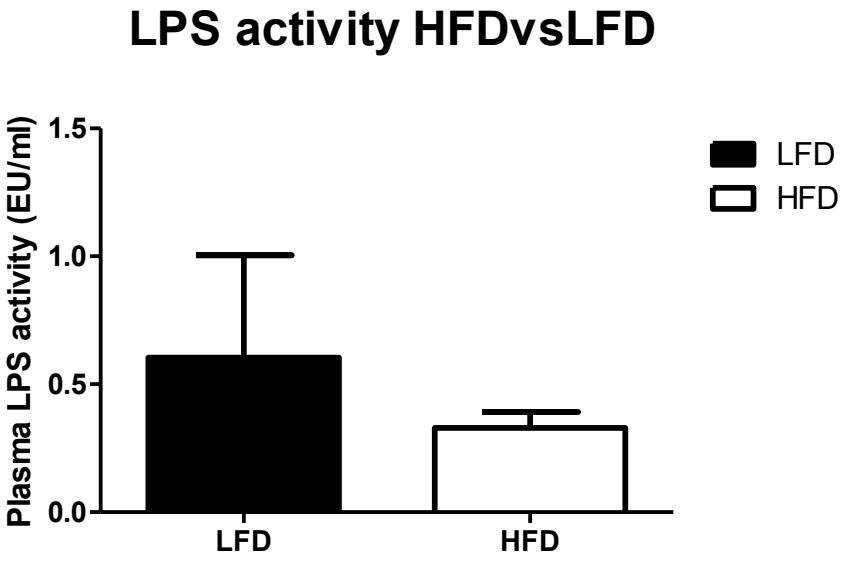


Figure 3.4 High fat diet did not alter LPS levels in circulation. At PND 84, no significant difference observed for plasma LPS activity between the HFD group and LFD group ($P = 0.44$). Data are presented as mean ± SEM.

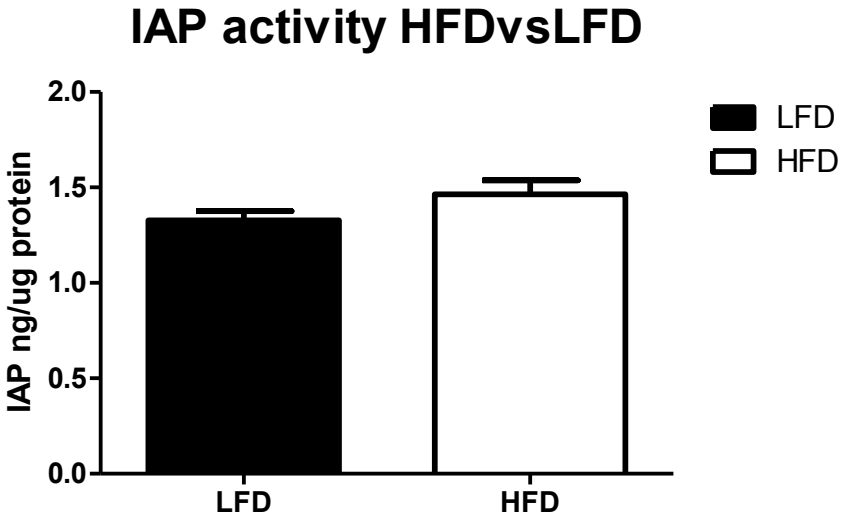


Figure 3.5 Activity of intestinal alkaline phosphatase in ileal mucosa at PND 84. There was no significant difference between two treatments ($P = 0.15$). Data are presented as mean ± SEM.

- Chapter 4: General Discussion

It has long been considered that the global pandemic of obesity and T2D is largely caused by westernized diet, genetic background and sedentary lifestyle, which lead to an imbalance of dietary energy intake and energy expenditure. However, several epidemiological studies have correlated antibiotic exposure in infancy with long-lasting metabolic syndrome later in life. We hypothesized that this was through the changes of gut microbiome induced by antibiotic exposure early in life and programmed the alterations in pancreas and intestine that predispose to the development of metabolic syndrome and a westernized diet could possibly faster the occurrence of metabolic syndrome. Experimental evidence for the relationship has not been provided. The overall objective of this study was to explore the association between early life antibiotic treatment and modified programming of gastrointestinal tract and pancreas development and provide direct experimental evidence for epidemiological studies.

4.1 Significance of the Study

In recent years, gut microbiota has become the subject of extensive research as an important environmental factor that is associated with pathogenesis of several diseases including metabolic syndrome, autoimmune diseases and neurological disorders. Although our knowledge of the commensal bacteria and their functions is growing rapidly, little is known about how perturbation of gut microbiota during the complex process of microbial colonization contributes to the disease development in the host. The intestinal microbiota is mainly acquired in the first few days after birth from maternal birth canal or the environment. The microbial diversity increases toward an adult-like microbiota in 3-5 years (Rodríguez et al., 2015). The early period is especially important when gastrointestinal tract and immune system are still developing and being shaped by gut microbiota colonization (Zeissig et al., 2014).

Since developed in 1940s, antibiotics and other antimicrobial agents are frequently prescribed for infants and children (Trasande et al., 2013). Although antibiotic treatment is critical for some pathogenic infections, several epidemiological studies have addressed the adverse effects of antibiotic exposure in the early period on the development of metabolic diseases later in life (Trasande et al., 2013; Bailey et al., 2014; Azad et al., 2014). Animal studies also provide evidence that antibiotics treatment to the host later in life exerts less alterations to the gut microbiota and subsequent metabolic outcomes compared with *in utero* administration (Cho et al., 2012; Cox et al., 2014; Schokker et al., 2015). With much attention paid to the disrupted microbiota and metabolic diseases, research is mainly focused on microbial fermentation that contributes to energy harvest and adiposity of the host. However, little is known about the relationships between gut microbiota and pancreatic islet development, which is vital to the regulation of glucose metabolism and impaired pancreatic β-cell functioning is a prerequisite of diabetes mellitus. The purpose of this thesis has been to identify the effects of early life antibiotic exposure on the intestinal microbiota and explore the subsequent impacts on developmental plasticity of the pancreas as well as the potential pathways that result in metabolic disease. To detect short and long-term effects of antibiotics in the gut, we examined the microbial community and measured important metabolites LPS and SCFA levels in the intestine, tested the response of host defense enzyme IAP and quantified expression of genes related to SCFA signaling. In addition, our study firstly determined the alterations in the development of pancreas in response to early life antibiotic exposure by GSIS and pancreatic islets immunofluorescence as well as identifying genes that are involved in this process. To further study whether antibiotics exposed in early life enhance the diet-induced obesity, we also developed a diet-induced swine model for children or adolescent obesity to provide experimental evidence for this relationship.

4.1.1 Achievement of Understanding the Effects of Antibiotics on Host Development

Our previous understanding of the effects of antibiotic-induced dysbiosis on metabolic syndrome pathogenesis was heavily based on epidemiological studies. However, the exact molecular relationship among microbe-derived gut metabolites, host signaling pathways and host physiology cannot be elucidated by epidemiological investigations. In the present study, we provided some potential mechanisms related to pancreatic development to explain the phenomenon for the first time. We developed a swine model for human infants with early exposure to amoxicillin and examined both microbial changes and host responses. With therapeutic doses of amoxicillin, the first observation is that two weeks of continuous oral amoxicillin administration did not lead to substantial global changes in microbial population in either the ileum and distal, which is consistent with previous studies in mice and pigs (Cox et al., 2014; Schokker et al., 2015). However, we identified two families, Enterobacteriaceae and Erysipelotrichaceae, that were enriched in antibiotic treated group at 7 days and the difference was not observed in the later time.

The piglets used in this study antibiotics were orally administered that would match infant antibiotic regimens, co-housed and fed on sow milk together to mimic the infant raising environment. Amoxicillin used in this study is a broad spectrum antibiotic that is commonly used to treat infant infections. It was summarized in previous studies that amoxicillin increases the number of Enterobacteria (Jernberg et al., 2010). In addition, Enterobacteriaceae includes many members that carry antibiotic resistance genes, which explains why this family enriched after amoxicillin exposure (Paterson et al., 2006). Erysipelotrichaceae was reported to be related to diet-induced obesity (Turnbaugh et al., 2008). In germ-free studies, it was demonstrated that the adiposity enhancement effects were only due to the changes in the microbiota, rather than direct effects of antibiotics. Germ-free mice that received microbiota from mice exposed to early life antibiotics showed similar adiposity phenotype as the donor (Cox et al., 2014). In addition to the differences observed

between the treatment groups, the analysis of generated bacterial profiles revealed that gut microbiota showed tissue specific traits and changed over time. Mostly, in mouse studies, antibiotics are administered to the dam, rather than the offspring so as to study the effect of *in utero* maternal exposure to antibiotics on next generation health. Besides prenatal period, early postnatal period is also a critical window for host development. Compared with mice, swine models make it possible to deliver antibiotics directly to newborns rather than though the mother which can guarantee the amount of antibiotics exposed to each individuals.

Our study firstly revealed that postnatal antibiotic exposure for the first 2 weeks could alter development of pancreatic islets and lead to impaired glucose metabolism regulation, suggesting the programming of certain microbes on host development may have long-lasting effects, although antibiotic administration stopped and the microbial difference between the two treatment groups was gone by that time. Furthermore, our study confirmed the hypothesis that disrupted microbiota is not only the consequences of metabolic disease; altered microbiota induced by antibiotics is also able to induce metabolic disease eventually (Bäckhed et al., 2009; Zhao et al., 2013). GSIS in isolated islets and immunostaining of pancreatic islets sectioning demonstrated the reduced glucose tolerance during OGTT was associated with changes in β-cell development and function. Pancreas undergoes postnatal development and substantial remodeling occurs in neonatal life (Hill et al., 2000). Therefore, important disturbance that influence β-cell maturation and neogenesis in early postnatal period could lead to long-term consequences. The altered pancreatic islets development observed in antibiotic treated pigs was accompanied by aberrant gene expression of a critical transcription factor *PDX-1* and overexpression of *IGF-2*, both of which are essential for glucose homeostasis and pancreatic development (Brissova et al., 2005; Casellas et al., 2015). Remarkably, our results suggest that in response to antibiotic treatment in the first two weeks, islets from pigs treated with antibiotics experienced higher functioning of β-cell insulin secretion ability at weaning but showed impaired glucose

control during OGTT later in life. Body weight changes were not observed in amoxicillin treated pigs throughout the whole experiment, suggesting that the altered metabolic changes in pancreas could appear before the obesity phenotypes. In fact, we did not measure adiposity composition of the pigs. It is very likely that antibiotic treated pigs have higher level of adiposity without difference in body weight as reported by a mouse study (Cho et al., 2012).

To further explore how the altered gut microbial composition in early period induced pancreatic development, we examined the microbial products and demonstrated an increase in circulating LPS and lower SCFA levels in antibiotic treated pigs. LPS is the major component of gram-negative bacteria and is able to trigger systemic inflammation by activating toll like receptors on cell membranes (Eizirik et al., 2009). Continuous infusion of LPS is able to induce glucose intolerance and substantial body weight gain (Cani et al., 2007; Nguyen et al., 2014). The enriched family Enterobacteriaceae in antibiotic treated pigs contributes to the enteric pool of LPS, which may initiate the inflammation in adipose tissue and islets of Langerhans that contributes to obesity pathogenesis. The increased LPS level in antibiotic group was accompanied with lower level of IAP, a brush border enzyme that functions to dephosphorylate LPS (Buchet et al., 2013), suggesting the inability of the host to suppress inflammation in pancreatic islets or adipose tissues when being challenged with higher concentration of LPS. The underlying mechanisms remain to be elucidated. There have been debates on the function of SCFA, another important microbial metabolite, which both contributes to energy harvest and serves as signaling molecules to induce satiety and enhance gut hormones production, including PYY and GLP-1. GLP-1 is known to be associated with β-cell homeostasis (Buteau et al., 2003; Brubaker et al., 2004). Our results suggest that antibiotic induced microbial changes lead to decreased SCFA in large intestine, which resulted in impaired protection of pancreatic development. In summary, the impaired pancreatic islets were likely due to the higher risk of inflammation and lower protection from gut enzymes and

hormones.

Our experimental results confirmed the epidemiological studies that early postnatal antibiotics induced gut microbial composition can result in metabolic disease. The importance of gut microbiota on metabolic disease was only recognized in the last 10 years and there were few studies on early life antibiotics and later life metabolic disease pathogenesis. Studies on environmental factors for metabolic disease were also mainly focused on diet-induced animal models and *in utero* environmental changes. In this study, we firstly pointed out the impaired β-cell function as the missing link between the disrupted gut microbiota in early postnatal period and the disease state later in life. Results in the present study suggest a careful use of antibiotic in human infants, especially during the first few months not just to avoid antibiotic resistance but also to reduce the risk for the development of obesity and other related symptoms later in life. Narrow-spectrum antibiotics that target specific pathogens will be more attractive compared with broad-spectrum antibiotics like amoxicillin so as to limit the broad effects on gut commensal bacteria. In addition, results in this study also indicate the potential for restoring gut microbial homeostasis shortly after antibiotic treatment to ameliorate the side effects of antibiotics on severe pathogen infections. These could be obtained by administrating probiotic capsules or enhancing residential bacteria communication by chemical signals like AI-2. Further investigations should be done to explore whether epigenetic modification is involved in the early life programming and the communications between gut microbiota and the host.

4.1.2 Achievement of developing a Diet-induced Swine Model for Obesity

In this study, we successfully developed a diet-induced swine model for metabolic disease. Pigs fed with high fat diet for 5 weeks showed substantial increase in body weight gain and adiposity, indicated by drastic increase in back-fat thickness. The

most frequently used animals for diet-induced obesity models are rodents. Murine models made great contributions to understanding the changes of host physiology and lipid metabolism to study high fat as the non-genetic factors for obesity development. However, due to the physiological differences between rodents and human beings, translational experiments for human obesity have many problems, which require the development of other animal models that are phylogenetically closer to human and share greater physiological similarities. Therefore, we developed a diet-induced swine model using commercial crossbred pigs to mimic the natural progress of obesity development instead of using genetic modified animals or using drugs like streptozotocin to induce β-cell damage. Impaired glucose tolerance showed up within 4 weeks of high fat feeding, accompanied with lower insulin secretion to the blood. Pigs on high fat diet failed to produce sufficient insulin when challenged with higher concentration of glucose, indicating irreversible damage in insulin secretion β-cell of pancreatic islets and relevant metabolic pathways.

In summary, our experimental results support the epidemiological studies that early postnatal antibiotic exposure can result in metabolic alterations. We provided the first piece of experimental evidence linking therapeutic antibiotics in the early life period to islet development and function later in life. In addition, we developed a diet-induced obesity model that can be used to explore the effects of early life antibiotics in the pathogenesis of diet-induced metabolic disease.

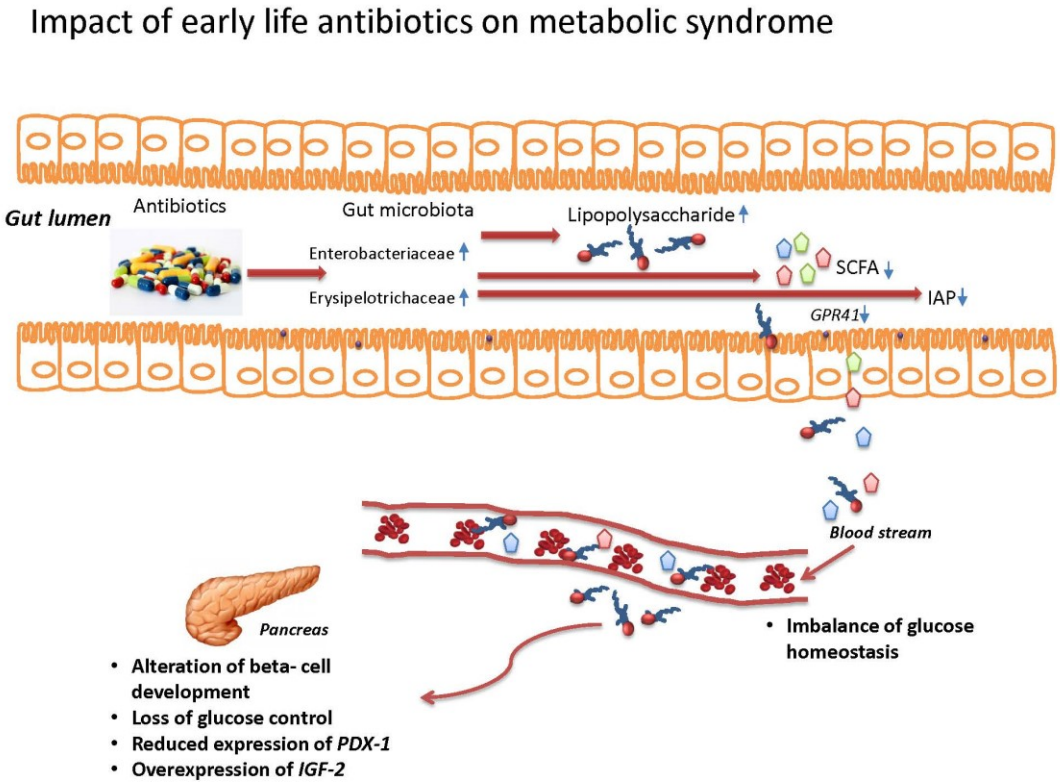


Figure 4.1 Overview of the achievements of this study. Early life antibiotic exposure in the first two postnatal weeks induced enrichment of two families of gut microbiome which might be responsible for the changes of LPS and production of SCFA and further influenced the level of intestinal alkaline phosphatase (IAP) and expression of SCFA receptor *GPR41*. The changes in the gut were accompanied with glucose intolerance and alteration of pancreatic beta cell function and development. This might be triggered by the changes of expression of two key genes *PDX-1* and *IGF-2* that are critical for the pancreatic development and function.

4.2 Future Directions

Our study contributed fundamental knowledge regarding commensal bacteria disruption and onset of metabolic disease, as well as altered pancreatic islet development involving in this process. However, there are a few limitations of the present study:

1. Amoxicillin induced microbial changes in the present study do not represent the effects of other commonly prescribed antibiotics. The extent of alterations

induced by antibiotics is dependent on the spectrum of the agent, dosage, duration of the treatment and the route of administration (Jernberg et al., 2010). The influence of other antimicrobial agents on obesity development and other related symptoms should be considered for future studies. It should also be noted that while changes in microbial populations were observed, it has not been demonstrated that these particular changes are causal. Given the fact that functions of each microbe are still not well explained and redundancy in microbial communities exits (Allison et al., 2008), simply reducing or increasing one or two families may not make a difference. As for the therapies to eliminate early life antibiotic adverse effects, it will be interesting to examine whether transplanting healthy gut microbiota from control group or from preserved bacteria before the antibiotic treatment to the treated pigs in the early period could restore or alleviate metabolic syndrome. In addition, methods that help promote microbial interaction and foster a healthy community of gut microbes should be considered as therapies to eliminate the side effects as quorum sensing molecule AI-2 has been shown to promote microbial balance after antibiotic exposure.

2. The alterations in metabolic pathways should be further explored in the future studies. The pancreatic islets in ANTI pigs underwent a process of hyper-functioning to normal functions from weaning to 7 weeks. How antibiotic-induced dysbiosis in the gut alters pancreatic development remains unknown. Although we identified *PDX-1* and *IGF-2* as potential factors that may contribute to altered β-cell functioning, they may instead represent a response to microbial-induced inflammation or other potential events as a result of early life antibiotic exposure. Whether inflammation caused by higher LPS in the blood is the major inducer for β-cell damage was not well studied in the present study. Early infusion of sub-therapeutic dose of LPS may help us understand the role of LPS in the process of pancreatic

development. Furthermore, what caused the inability of the host to secret sufficient IAP is still a mystery. In future studies, immune cell profile and cytokines status should be examined to reflect the inflammation state in treatment groups. In this study, we mainly focused on the metabolic changes in pancreas and intestine. It will be of great interest to determine the lipid metabolism in liver and adipose tissue as it was reported that disrupted microbiota altered hepatic metabolism of fatty acids and lipids (Cho et al., 2012). To better describe metabolic disease, other symptoms including high blood pressure and dyslipidemia should be examined. Lower level of SCFA was observed in ANTI group, accompanied with lower expression of SCFA receptor *GPR41*. SCFA induce satiety by promoting secretion of gut hormones. As lower SCFA detected in ANTI group, we expect a lower secretion of GLP-1 and PYY in the antibiotic exposed pigs. Therefore, GLP-1 levels and SCFA signaling (e. g. the role of GPR41 in energy expenditure) in the antibiotic treated animals should be explored in the future study.

3. The approaches applied for measuring feed intake and back-fat thickness can be improved in following studies. The method for determining back-fat thickness cannot precisely measure fat profile. Instead, it was only used to estimate the adiposity status in pigs. In mice studies, dual energy X-ray absorptiometry (DEXA) scanning is being used to assess adiposity (Chen et al., 2012). However, only few studies have used this method to assess visceral fat in pigs. It will be of great interest in using this method in our swine model to accurately measure the fat composition in the future. In addition, the method used for measuring feed intake should be replaced with a computerized feed intake recording system (Hyun et al., 1997) to better record energy consumption. Small sample size and large variation in an outbred population was a limitation of these animal studies. A larger study population will be important to increase the power of future studies.

4. Determining antibiotic effects on high fat diet induced obesity. In chapter 2, we demonstrated that early life antibiotics lead to metabolic changes in later life. In the chapter 3, we succeeded in establishing a diet-induced swine model. As a strong factor for obesity, high fat diet alone can induce metabolic phenotypes (Turnbaugh et al., 2008). However, the actual progress of development in human beings is more complex and involves many environmental factors, which can worsen the situation when added up. It was previously reported that early life antibiotic exposure enhanced the influence of HFD induced obesity in a mouse model (Cox et al., 2014). Therefore, we expect exacerbated metabolic symptoms in the antibiotic treated pigs when challenged with HFD feeding. It will be of great interest to examine the microbial changes throughout the whole experiment and determine how the altered gut microbiota response to high-fat diet challenging later in life. The combination of the two may better explain the high prevalence of obesity in western countries since prescription of antibiotics and consumption of HFD is frequent in these countries. Although the pig model we describe here is not perfect, we believe that it offers significant advantages over rodents for basic and translational research regarding the physiological similarity between human and porcine GI tract and pancreatic islets.

5. Studying epigenetic effects of antibiotic on microbial changes and genes related to metabolic disease. Epigenetic programming is one important mechanism through which early environmental exposures affect chronic disease development by impacting gene expression through DNA methylation, histone or chromatin modifications and regulation of microRNAs. There are several examples indicating that early life microbial composition impacts long-term physiology. One example was the delivery of *E.coli* enhanced the effects of high-fat diet fat on pad accumulation in offspring from HFD fed

mothers (Fåk et al., 2012). Resident bacteria were also reported to be involved in epigenetic regulation of IAP through butyrate, one of SCFA (Hinnebusch et al., 2003). In addition to the regulation of IAP, SCFA are also involved in *GPR41* methylation in obese and T2D patients through regulation of histone deacetylases (Remely et al., 2014). In obese and diabetic patients, epigenetic modifications of transcription master regulators were identified including *PDX-1* and *IGF-2* (Park et al., 2008; Volkmar et al., 2012). Therefore, the metabolic phenotypes observed later in life could be due to the early programming of antibiotic treatment. It will be of great interest to examine the epigenetic modification of the differentially expressed genes in pancreas and intestine as well as the genes related to microbial metabolites fermentation and detoxification to determine the signaling pathways that the altered microbiota programmed the metabolic syndrome in human infants.

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