Long-term effects of early-life gut microbiota perturbations on pancreatic islet development

by

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Abstract

Impaired secretion of insulin from pancreatic β-cells, along with loss of cell mass, is one of the hallmarks of the pathogenesis of type 2 diabetes, a metabolic disorder characterized by chronic hyperglycemia. In humans, β-cells' ability to secrete insulin in response to glucose is acquired in the first year of life and is considered as a critical window for β-cell mass expansion and maturation. However, early exposure to environmental and metabolic stressors could impair the acquisition of β-cell functional maturation and increase the susceptibility to develop diabetes. Along with genetic factors, lifestyle and diet, the gut microbiota has been considered as an environmental factor that plays an important role in the etiology of diabetes. Children treated with more than one course of antibiotic during the first 2 years of life have a higher risk of childhood overweight, which is a risk for developing diabetes. Moreover, previous work by our laboratory found that antibiotic-induced gut microbiota perturbations altered pancreatic islet function and morphology in weaning piglets after antibiotic withdrawal, with impaired glucose tolerance later in life.

Therefore, the first aim of this thesis is to examine earlier, neonatal pancreatic islet functional maturation during antibiotic administration in the piglet model with the hypothesis that antibiotic administration will change the composition of the gut microbiota, leading to alterations in microbial metabolites that will be associated with impaired β-cell functional maturation.

We examined the gut microbiota composition and pancreatic islet function and growth from neonatal piglets exposed to antibiotics. Compared with controls (CON), antibiotic-treated (ANTI) postnatal day (PND)7 pigs had elevated transcripts of proteins involved in GLP-1 synthesis or signaling in islets ($p<0.05$) coinciding with a pattern of higher plasma GLP-1 ($p=0.11$), which were not detected by PND14. mRNA levels of *Tnf* ($p<0.05$) a pro-inflammatory cytokine, and *Npg1*

($p<0.05$), a cathelicidin, were also transiently increased in pancreas of PND7 ANTI pigs' concomitant with 10-fold higher culturable intestinal coliforms ($p<0.05$). Antibiotic-induced changes in ileal microbial composition at PND7 included relative increases in genera *Escherichia, Coprococcus, Ruminococcus, Dehalobacterium,* and *Oscillospira,* which were normalized after antibiotic withdrawal. In ANTI islets at PND14, the expression of key regulators *Pdx1*, *Igf2* and *Tcf7l2* was down-regulated, preceding a 40% reduction of β-cell area ($p<0.01$) and insulin content/islet at PND49 ($p<0.05$). At PND49, a 2-fold elevated non-fasted plasma insulin concentration ($p=0.07$) was observed in ANTI compared with CON.

In conclusion, antibiotic treatment of neonatal piglets elicits gut microbial changes accompanied by phasic alterations in key regulatory genes in pancreatic islets at PND7 and 14. By PND49, reduced beta-cell area and islet insulin content were accompanied by elevated non-fasted insulin despite normoglycemia, indicative of islet stress.

The second aim was to study whether elevated gut *Escherichia coli* in combination with antibiotics contributed to altered glucose tolerance in adulthood. We used C57BL/6 black mice exposed to oral doses of amoxicillin during the first 14 days of life in the absence or presence of *Escherichia coli* in the gut, and then weaned onto a high-fat diet. We observed an increase in body weight in *Escherichia coli* mice at PND14. Combined exposure to *Escherichia coli* and early-life antibiotic treatment caused worsened glucose tolerance at age 7 weeks, consistent with insulin secretion dysfunction and/or insulin resistance.

Together, this work confirms that gut microbiota perturbations elicit long-term effects on β-cell function and glucose homeostasis. Possible mechanistic explanations are likely related to alterations in the temporal programming of transcription factors important for controlling β-cell mass and function.

Preface

This thesis is an original work by Carla Sosa Alvarado. The research project, of which this thesis is a part, received ethics approval form the University of Alberta Research Ethics Board, Project Name "Defined microbial communities", AUP 00000671. I was funded by CONACyT Graduate Students Award for International Studies from Mexico's Government, and Women and Children's Health Research Institute (WCHRI).

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Chapter Three authors contribution: conception and design of experiments: Catherine B. Chan and Benjamin P. Willing. Performance of the experiments: Carla Sosa Alvarado, Nicole Coursen, Tinting Ju, and Andrew Forgie. Analysis of the data: Carla Sosa Alvarado. Contribution of reagents/materials/analysis tools: Carla Sosa Alvarado, Benjamin P. Willing and Catherine B. Chan.

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ANTI: Antibiotic

βIGF2KO: Beta-cell specific IGF-2 knockout mice

FFAR: Free fatty acid receptor

GAP: GTPase-activating protein

GCG: Glucagon

GLP-1: Glucagon-like peptide 1

GLP-1R: GLP-1 receptor

GLUT: Glucose transporter

GPCR: G-protein couple receptor

GSIS: Glucose-stimulated insulin secretion

GWAS: Genome-wide association studies

HOMA-IR: Homeostatic Model Assessment of Insulin Resistance

IFP: impaired fasting plasma glucose

IGF-2: Insulin growth factor 2

IGT: impaired glucose tolerance

IL: Interleukin

INS: Insulin gene

IRS: Insulin receptor substrates

ISL-1: Insulin gene enhancer protein

K_{ATP}: ATP-sensitive potassium channels

MAFA: V-maf musculoaponeurotic fibrosarcoma

MCT-1: Monocarboxylate transport 1

MODY: Maturity-onset diabetes of the young

NEUROD1: Neurogenic differentiation 1

NKX6.1: Homeobox protein Nkx-6.1

NOD: Non-obese diabetic

OGTT: oral glucose tolerance test

PDX-1: Pancreas/duodenum homeobox protein-1

PI3K: Phosphatidyl inositol-3 kinase

PIP-2: Phosphatidylinositol (PIP)-2

PKB: Protein kinase B

PND: Postnatal day

PSCK: Prohormone convertase

SCFA: Short-chain fatty acids

SMCT-1: Sodium dependent monocarboxylate transporter-1

SNP: Single nucleotide polymorphisms

TCA: tricarboxylic acid

TCF7L2: Transcription factor 7 like 2

TNF: Tumor necrosis factor

TLR: Toll-like receptors

UCN3: Urocortin

Chapter 1. Literature Review

1.1. Diabetes mellitus

Diabetes is a chronic metabolic disorder characterized by an altered metabolism of nutrients that results in insulin resistance, impaired insulin secretion or a combination of both (DeFronzo et al., 2015). Diabetes is a leading cause of kidney failure, neuropathy, cardiovascular disease, and retinopathy, all of which are major causes of premature death and early disability (Diabetes Federation International, 2019; Zheng et al., 2018) Currently, diabetes affects 415 million adults (type 2 diabetes represents 90% of the cases) and will increase to 642 million by 2045 (Diabetes Federation International 2019). 8.4% of all deaths are attributed to type 2 diabetes-associated complications and impact significantly health-services and resources (Nanayakkara et al., 2021). In Canada, the rates of diabetes are predicted to increase to 12.1% of its population by 2025 (Houlden, R.L, FRCPC, 2018). One in 10 deaths of Canadian adults was attributed to diabetes-related complications in 2008/09, suggesting that diabetes and its complications are a serious chronic condition (Houlden, R.L., FRCPC, 2018).

To mitigate the negative impacts of diabetes, the implementation of lifestyle interventions, medications, and behaviour changes are effective therapeutic strategies to reduce hyperglycemia and delay the onset of diabetes (Taylor & Barnes, 2018). However, the mechanisms underlying the initial stages of diabetes remain incompletely understood. In the past 10 years, studies have recognized that significant changes in pancreatic β–cell function may be the central cause of diabetes, however observations in the interaction of the pancreas with other organs must be considered.

1.1.1. Type 2 diabetes pathophysiology. Type 2 diabetes is characterized by the dysregulation of nutrient metabolism due to an inadequate response by insulin-dependent tissues

to circulating insulin at physiological levels (insulin resistance) and/or a progressive and gradual loss of pancreatic β–cell function (impaired insulin secretion) (Esser et al., 2020; Pearson, 2019; Zhong & Jiang, 2019a). Prediabetes is a period characterized by mild elevation of glucose concentration in the fasting state (5.6 to 6.9 mmol/l) and 2-hours (7.8-11.0 mmol/l) during an oral glucose tolerance test (OGTT), and or elevated HbA1c (39 to 46 mmol/mol). Clinical biomarkers have been used to diagnose diabetes in a wide spectrum of ages, based on blood glucose criteria, either impaired fasting plasma glucose (IFP) concentration that is greater than 7 mmol/L, and HbA1c (39-46 mmol/mol) and impaired glucose tolerance (IGT) according to American Diabetes Association (American Diabetes Association, 2020).

1.1.2. Causes of type 2 diabetes. However, type 2 diabetes etiology is multifactorial as it depends on genetic and environmental component interactions. Type 2 diabetes risk attributed to genetic inheritance is about 30%-70% (Adams & Vella, 2018; Merino et al., 2017) with a higher rate in identical twins (60-90%) in contrast to non-identical twins and siblings (Kleinberger et al., 2018). The first genetic studies in type 2 diabetes were performed using candidate genes and linkage analysis that confirmed the association of genetic markers with type 2 diabetes susceptibility (Barroso et al., 2003) in various populations, however, the replication and consistency of the results were limited (Witka et al., 2019). After human genome was sequenced, the genome-wide association studies (GWAS) were developed, and a large number of gene variants, also called single nucleotide polymorphisms (SNPs), associated with type 2 diabetes were identified in case-control populations from different regions and ethnic groups (Hu et al., 2020; Merino et al., 2017; Szabo et al., 2018).

To explore the physiological relevance of genetic variants, GWAS analysis was correlated with metabolic traits manifested in type 2 diabetes. The results of multiple studies revealed that

most of the genetic variants were associated with abnormal function of transcription factors. These transcription factors are more involved in β–cell function than insulin action (Nayak et al., 2021; Salunkhe et al., 2018). In the last decade, more than 144 genetic variants have been discovered, however, they confer small (~10%) but significant effect of diabetes heritability, implying that other factors contribute to the nature of diabetes (Doliba, 2018; Szabo et al., 2018; Xue et al., 2018).

1.1.3. Monogenic forms of diabetes. The functional relevance of these genetic variants can be observed in the main two monogenic forms of diabetes: neonatal diabetes and maturity-onset diabetes of the young (MODY) (Ganesh et al., 2017; Greeley et al., 2011). Both types of monogenic diabetes account for 1-5% of individuals with diabetes and are characterized by early-onset of diabetes (>6 mo for neonatal diabetes and before the age of 40 years for MODY) (Ganesh et al., 2017; Kleinberger et al., 2018; Weinreich et al., 2015). In contrast to type 2 diabetes, monogenic diabetes arises from a mutation in an heterogenous group of genes that leads to a severe disruption of the β-cell function and development (Jennings et al., 2020; Kleinberger et al., 2018). For instance, mutations of pancreatic and duodenal homeobox 1 (*PDX-1*) (MODY4) and neurogenic differentiation-1 (*NEUROD1*) (MODY6) are described in MODY (Hattersley & Patel, 2017). Both Pdx-1 and NeuroD1 are essential transcription factors for the development of the pancreas at early stages, differentiation of endocrine-cell lines from pancreatic precursor cells, and insulin gene expression (McKinnon & Docherty, 2001; Fujimoto & Polonsky, 2010; Rickels et al., 2020).

1.1.4. The role of environmental factors in type 2 diabetes etiology. Besides genetic factors, environmental factors play an important role in the etiology of type 2 diabetes (Lascar et al., 2018). A diet with high-calorie content and sedentary lifestyles are overwhelmingly associated

with obesity and insulin resistance (Esser et al., 2020). Obesity is defined as the excess body fat weight with a body-mass index greater than 30 Kg/m2 and is commonly known as a risk factor for type 2 diabetes (Diabetes Federation International 2019). During the emergence of obesity, fat accumulation in liver, pancreas, and skeletal muscle impairs glucose uptake leading to the appearance of insulin resistance (Eckel et al., 2011; White et al., 2016). The abnormal lipid metabolism seen in obesity leads to an elevated production and release of free fatty acids in circulation that, in combination with hyperglycemia, trigger inflammatory signaling in β-cells (Ortega-Camarillo, 2019). For instance, the accumulation of saturated fatty acids, such as Palmitate, induces the production of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), which are proinflammatory cytokines that downregulate key β-cell genes involved in insulin synthesis and identity (Nordmann et al., 2017). Elevated circulatory free fatty acids together with hyperglycemia exacerbate the loss of β-cell function by inducing endoplasmic reticulum stress and mitochondria dysfunction, important intracellular organelles for glucose-stimulated insulin secretion (GSIS) (Eckel et al., 2011; Prentki et al., 2020).

1.2. Islets of Langerhans. The islets of Langerhans are specialized endocrine cells that play an important role in glucose homeostasis (Cohrs et al., 2020; Weir & Bonner-Weir, 2013). In healthy adults, the islets of Langerhans constitute between 1-2% of the total volume of the pancreas and contain (Paniccia & Schulick, 2016) the major hormone-producing endocrine cells, which are β, α, δ, ε and PP cells that are responsible for the production of insulin, glucagon, somatostatin, ghrelin and pancreatic polypeptide (Ruchelli & Bhatti, 2019). In humans, pancreatic β-cells constitute the major volume of islet endocrine cells (60%), with smaller proportions of α-cells (30%), δ-cells (10%) and pancreatic polypeptide and ghrelin producing cells (1%) (DeFronzo et

al., 2015). Pancreatic β-cells are major regulators in the maintenance of proper glucose concentration after a meal and during fasting state by their ability to produce insulin.

1.2.1. Insulin. Insulin is an anabolic hormone that stimulates metabolic fuels storage and maintains glucose homeostasis (Campbell & Newgard, 2021). The biosynthesis of human insulin begins with the transcription of preproinsulin mRNA from the insulin gene (*INS*) in humans, or *INS1* and *INS2* genes in mice (Tokarz et al., 2018). Preproinsulin is translocated to the endoplasmic reticulum where its signal sequence is cleaved by signal peptidase to generate proinsulin. Proinsulin consists of a single chain of 86 amino acids and three disulfide bonds that stabilize the folded conformation (Ilie, 2020). The transit of proinsulin to the Golgi network is followed by prohormone convertase 1 and 2-mediated cleavage of the central fragment of the proinsulin (C-peptide). The removal of C-peptide leads to the formation of mature insulin, which is constituted by two insulin peptides (chain A and B) linked by two disulfide bonds (Zhou et al., 2013). The newly formed mature insulin is stored as a hexamer in secretory vesicles inside the β-cells. In response to stimuli, insulin hexamer is secreted and dissociates in the blood stream as monomers, which binds to insulin receptors present in different tissues and initiates insulin-associated physiological effects (Haeusler et al., 2018; Tokarz et al., 2018).

In response to a prolonged glucose exposure, β-cells sustain insulin secretion in two phases. The first phase is the rapid response stimulated by glucose and consists of the release of insulin vesicles pre-docked at the cell membrane. The second phase is stimulated by nutrients, hormones, and incretins, and induces the mobilization of insulin-containing granules from the intracellular pool to the cell membrane (Tokarz et al., 2018). The mechanisms characterized in each insulin secretion phase will be explained in the following paragraph.

1.2.2. Glucose-stimulated insulin secretion. Glucose is the main metabolic signal that stimulates insulin secretion. Changes in glucose concentration in blood are tightly linked to β-cell glucose metabolism and insulin output (Campbell & Newgard, 2021). Glucose enters the intracellular space of the β-cells through glucose transports GLUT1 (humans) or GLUT 2 (mouse) that is expressed in the cell membrane. Intracellular glucose is phosphorylated by glucokinase, also known as hexokinase IV, to produce glucose-6-phosphate (Boucher et al., 2014). The low affinity of glucokinase for glucose ensures intracellular glucose concentration matches the extracellular concentration (Campbell & Newgard 2021). Glucose-6-phosphate enters glycolysis to produce pyruvate, which is incorporated in the mitochondrial tricarboxylic acid (TCA) cycle in the mitochondria. The metabolism of glucose in the mitochondria leads to a chain of events: an increase in the ATP/ADP ratio that promotes the closure of ATP-sensitive potassium channels (K_{ATP}) that leads to cell membrane depolarization. Voltage-gated L-type and calcium (Ca^{2+}) channels open in response to the cell membrane depolarization and the increase in intracellular calcium concentration that triggers insulin exocytosis (Campbell & Newgard, 2021)

1.2.3. Amplifiers of glucose-stimulated insulin secretion. Metabolites in blood circulation have an additional effect on glucose-stimulated insulin secretion which is independent of K_{ATP} channels (Rutter et al., 2020). It is estimated that K_{ATP} channel-independent/ amplifying pathway contributes between 60-70% of the total insulin secreted in response to glucose (Campbell & Newgard 2021). In addition to glucose, other dietary nutrients such as free fatty acids, bile acids and amino acids induce the secretion of incretins, such as glucagon-like peptide 1 (GLP-1) (Müller et al., 2019). GLP-1 increases insulin secretion in a glucose-dependent manner and suppresses glucagon release from the pancreatic α-cells, assisting with the balance of the anabolic effects of insulin (Stephen & Ramracheya, 2018). Circulating GLP-1 accounts for about 40% of insulin

secretion despite its short half-life (1-2 minutes), which is due to rapid degradation by dipeptidyl peptidase 4 (DPP-4) (Müller et al., 2019).

1.2.4. Insulin signaling. Insulin performs its metabolic effects on tissues such as skeletal muscle, adipose tissue and liver through the activation of insulin receptors. The insulin signaling pathway activates a cascade of multiple proteins via phosphorylation.

The circulating insulin binds to its cell membrane receptor, a tetrameric protein from the family of tyrosine kinase receptors. The insulin receptor consists of two extracellular α subunits, which is the ligand-binding site, and two transmembrane β-subunits which contain tyrosine kinase domains (Petersen & Shulman 2018). Insulin binds to the α-subunits of the insulin receptor and induces a conformational change that activates the tyrosine kinase activity of β subunits. The kinase activity of the insulin receptor auto-phosphorylates tyrosine residues in the β subunits. The phosphorylated tyrosine residues are binding sites for the insulin receptor substrates (IRS), a family of scaffold proteins (Boucher et al., 2014). Once IRS is activated by phosphorylation, IRS proteins that bind to src-homology-2 domain proteins (SH2), such as the enzyme phosphatidylinositol 3-kinase (PI3K), or other proteins without enzymatic activity like adaptor protein Grb2. PI3K promotes the activation of the protein kinase B (PKB or AKT), which then phosphorylates GTPase-activating protein (GAP) AKT substrate of 160 kDa (AS160), that is bound to GLUT-4 vesicles. The role of AS160 is the translocation of GLUT-4 from the intracellular vesicles to the cell for glucose uptake (Petersen & Shulman, 2018; Tokarz et al., 2018).

1.3. β-cell mechanisms of adaptation during the onset of type 2 diabetes.

The proper activation of β-cell mechanisms allows the preservation of glucose homeostasis that can be sustained for decades and delay diabetes appearance (Cohrs et al., 2020; Eckel et al.,

2011; Prentki et al., 2020). During obesity, hyperinsulinemia is a β-cell adaptation response to nutrient-induced metabolic stress and positive energy balance (Esser et al., 2020; Ottosson-Laakso et al., 2017; Prentki et al., 2020; Weir, 2020). This long-term adaptive response requires changes in genes implicated in β-cell mass expansion, β-cell function and identity.

1.3.1. β-cell mass and type 2 diabetes. During the transition from the neonatal period to adulthood, β-cell proliferation is suppressed as the β-cells mature (Qiu et al., 2017). However, histological studies in the human pancreas identified that β-cell mass was increased approximately 20%-50% in subjects with obesity (Inaishi et al., 2016) while in subjects with type 2 diabetes, β-cell mass was significantly reduced (24% to 65%) (Cohrs et al., 2020).

In the last decade, it has been considered that β-cell mass expansion is an adaptation response to increased insulin secretion and overcome the metabolic demands induced by insulin resistance and hyperglycemia (Mosser et al., 2015; Weir et al., 2020; Wortham & Sander, 2016).

In obesity and prediabetes, chronic hyperglycemia and elevated free fatty acids trigger β-cell mass expansion, suggesting that environments rich in nutrients and stressors can induce β-cell proliferation later in life (Chen et al., 2017; Weir et al., 2020). While in type 2 diabetes, β-cell mass declines due to an enhanced rate of apoptosis but also due to a dedifferentiation process. Single-cell RNA sequencing analysis performed in islets of type 2 diabetic donors identified the upregulation of genes sets frequently expressed in toddlers or juvenile islets, suggesting that type 2 diabetes undergo a degree of dematuration (Arda et al., 2016; Avrahami et al., 2020). It remains unclear whether the loss of β-cell mass or β-cell identity contributes more to insulin deficiency during the first stages of type 2 diabetes pathophysiology (Cohrs et al., 2020; Taylor et al., 2019; Weir et al., 2020).

1.3.2. β-cell dysfunction during type 2 diabetes. Metabolic stress downregulates the expression of β-cell key transcription factors involved in β-cell differentiation and functional maturation (Marselli et al., 2020).

Pdx1 is one of the main transcription factors that is expressed in islet development during the fetal stage, and later is selectively expressed in adult mature β-cells (Rickels et al., 2020; Scharfmann, 2007). Heterozygous individuals that carry point mutations in the *PDX1* transactivation domain have an impaired GSIS. The contribution of *PDX1* mutations to β-cell dysfunction was found to be due to a low expression of downstream targets of Pdx1: *INS, MAFA, UCN3, ISL-1, and NKX6.1*, which are required for β-cell functional maturation (Wang et al., 2019). Taken together, these findings suggest that *PDX1* loss leads to impaired β-cell maturation and consequently increases the predisposition to diabetes (Rickels et al., 2020). After *PDX1* deletion in islets from adult mice, the expression of key regulators of glucose metabolism, *GLUT2*, *GCK* and *INS-I* and *INS-II* were depleted, while the expression of α-cell markers, *MAFB* and *GCG*, were increased. The long-term effect of *PDX1* loss led to constant hyperglycemia and diabetes development. Altogether, these experiments suggest that *PDX1* plays a significant role in the activation of genes sets related to β-cell function, and the loss of *PDX1* contribute to diabetes onset (Ahlgren et al., 1998; Gao et al., 2014).

Studies in population cohorts reported that *TCF7L2* variants were frequently identified in type 2 diabetes subjects, suggesting that *TCF7L2* loss confers risk for type 2 diabetes (Adams & Vella, 2018). The *TCF7L2* gene encodes for the transcription factor like-7 2 (*TCF7L2*), a transcription factor that regulates cell growth through the signaling Wnt pathway (Mitchell et al., 2015). *TCF7L2* upregulates proglucagon and GLP-1 receptor transcription, in both human and rodent islets (Adams & Vella 2018). Individuals with type 2 diabetes or those who carry *TCF7L2*

SNPs have impaired insulin secretion induced by GLP-1 (Pilgaard et al., 2009; Schäfer et al., 2007; Shu et al., 2009) and islets from type 2 diabetic donors have significantly reduced *TCF7L2* protein level (about 50% low) and a blunted response to GLP-1-induced insulin secretion due to a downregulation of GLP-1 receptor (da Silva Xavier et al., 2017; Lyssenko et al., 2007; Shu et al., 2009). The use of rodent models has contributed to understanding the mechanisms underlying impaired GLP-1-mediated insulin secretion cause by *TCF7L2* SNPs. Rodent models of type 2 diabetes (diet-induced obesity (DIO), leptin receptor deficient *db/db* mice, and Zucker rat) had a reduced expression of *TCF7L2* (Shu et al., 2009). Moreover, isolated islets from *TCF7L2*-null mice had impaired insulin secretion in response to high glucose concentration and GLP-1, impaired β-cell proliferation, and downregulation of *INS* and *MAFA* genes (Takamoto et al., 2014). Since most of the studies focused on understanding the unique role of *TCF7L2* gene expression and function, it is not known whether other tissues that express *TCF7L2* gene trigger compensatory mechanisms that regulate the *TCF7L2* gene expression and/or protein content to mitigate glucose intolerance and delay the appearance of diabetes. However, metabolic stressors, such as high-fat diet, can enhance the negative effects of *TCF7L2* variant on β-cell function, highlighting the important contribution of environmental factors to gene variants effect (da Silva Xavier et al. 2017; Shu et al. 2009).

Besides the effect of incretin on β-cells, IGF-2 is considered as a key regulator of β-cell mass expansion in response to metabolic stress (Zhong & Jiang, 2019a). IGF-2 knockout mice (βIGF2KO) under high-fat diet displayed lower insulin secretion and reduced β-cell mass suggesting that abnormal function of IGF-2 can increase the risk of β-cell dysfunction, which may progress to diabetes (Modi et al., 2015).

β-cells' mechanisms of adaptation to insulin resistance can be sustained for decades as it is observed that only 1/3 of the subjects with obesity progress to type 2 diabetes (Doliba, 2018; Gregg et al., 2012; Wortham & Sander, 2016)

Dietary interventions with calorie restriction showed that only 40% of the participants with type 2 diabetes recovered β-cell function as evidenced by sustained normoglycemia (Steven et al., 2016) suggesting that in addition to genetic and/or environmental factors, some individuals have a higher susceptibility to type 2 diabetes due to inadequate β-cell compensation upon nutrient-metabolic demands later in life (Swisa et al., 2017; Wortham & Sander, 2016).

1.4. Neonatal pancreatic β-cells functional maturation and development

Researchers in islet development have focused on understanding the steps and mechanisms that give rise to a fully functional mature β-cell, and potentially explain the causality of β-cell dysfunction and mass loss during different stages of diabetes pathophysiology (Ackermann & Gannon, 2007; Aguayo-Mazzucato et al., 2011; Avrahami et al., 2020). Also, perturbations during early life contribute to impaired β-cell growth and functional maturation acquisition that increase the susceptibility to diabetes at later life (Tixi-Verdugo et al., 2018). The steps of pancreatic β-cells development are described in the next paragraph.

1.4.1. Role of nutrients in neonatal β-cell. The development and differentiation of specialized endocrine cells initiates at the fetal stage, but β-cells become functionally mature during the first year of life in humans (Bonner-Weir et al., 2016; Martens et al., 2013; Stolovich-Rain et al., 2015). During the transition from birth to weaning, β-cells gradually become sensitive to fluctuations of glucose and secrete the proper amount of insulin to maintain glucose homeostasis, acquiring adult-like mature β-cells. Immature β-cells display a high insulin secretion in response to basal (2.7-5 mM) and small fold-increase in high (>10 mM) glucose concentration

exposure, which is adjusted to an adult-like response in an age-dependent manner (Aguayo-Mazzucato et al., 2006; Arda et al., 2016; Blum et al., 2012; Gregg et al., 2012; Helman et al., 2020; Qiu et al., 2017).

In addition to age effects, the nutrient environment is a key factor that contributes to the onset of β-cell glucose responsiveness and insulin secretion dynamics (Helman et al., 2020; Henquin & Nenquin, 2016; Qiu et al., 2017; Zeng et al., 2017). Newborn islets secrete insulin in response to glucose and amino acids, while in fetal islets insulin secretion is stimulated by amino acids, not glucose (Helman et al., 2020; Henquin & Nenquin, 2016; Zeng et al., 2017). This observation highlights that a shift in the nutrient environment promotes β-cell sensitivity to glucose concentrations which is a critical signature of mature β-cells.

The switch from fat-rich maternal milk to a diet rich in carbohydrates occurs at weaning and triggers β-cell mechanisms of adaptation and pathways that accelerate functional maturation (Jaafar et al., 2019; Stolovich-Rain et al., 2015). Mice that were under a prolonged suckling period had blunted β-cell replication in response to high glucose, high insulin response to basal glucose concentration, and downregulation of *INS1*, *INS2* and *MAFA* genes that are related to β-cell maturation (Jaafar et al. 2019; Stolovich-Rain et al. 2015). These findings suggest that weaning is a critical step for enhancing β-cell glucose-response that is sustained in adulthood.

1.4.2. Role of transcription factors in neonatal β-cells. Islet development and growth is highly regulated by a hierarchy of transcription factors identified in fetal and/or neonatal stages. The expression of transcription factors occurs in a unique pattern and time that is conserved across species. The transcription factors FoxA 1 and 2, Pdx-1, Neurogenin3, NeuroD1, Nkx2.2, Nkx6.1, Hnf4a and Pax4 are involved in β-cell differentiation (Conrad et al., 2014; Jennings et al., 2020; Tritschler et al., 2017).

MAFA is considered as a transcription factor that is expressed specifically in mature neonatal β-cells in different species (Aguayo-Mazzucato et al., 2011; Arda et al., 2016; Avrahami et al., 2020; Qiu et al., 2017; Tritschler et al., 2017; Zeng et al., 2017). *MAFA* is one of the major regulators of insulin gene expression and the glucose transporter GLUT2 (Jennings et al., 2020; Aguayo-Mazzucato et al., 2011). The low expression of *MAFA* has been identified in islets of embryonic humans and type 2 diabetes subjects, suggesting that *MAFA* expression is crucial for the maintenance of differentiated and functional β-cells (Peddinti et al., 2017; Rutter et al., 2020). The expression of *MAFB* is gradually lost in mature β-cells and is negatively correlated with *UCN3* expression, which is a marker identified only in mature β-cells (Avrahami et al., 2020; Qiu et al. 2017; Tritschler et al. 2017; Blum et al. 2012).

1.4.3. Role of incretins and growth factors in neonatal β-cells. The effects of GLP-1 on neonatal β-cell functional maturation and proliferation have been extensively studied with the aim to identify the mechanisms that ameliorate or prevent the development of diabetes. GLP-1 receptor, GLP-1 bioactive form and prohormone convertase have been detected in neonatal islets of human, pigs, and mice (Kim et al. 2020; Arda et al. 2016). Pcsk1/3 colocalize with GLP-1 bioactive form in α-cells of neonatal mice, suggesting that α-cells regulate β-cell proliferation at early age through the production of GLP-1 (Kilimnik et al., 2011).

Mouse or rat models of type 2 diabetes treated with GLP-1 or exendin-4 via intraperitoneal injections after birth showed an improvement in insulin secretion and β-cell mass, suggesting a protective effect of GLP-1 in β-cell function at early stages, even before the onset of diabetes (Tourrel et al., 2001; Portha et al., 2011). Furthermore, in seven-day-old neonatal Wistar rats treated with streptozotocin, GLP-1 exhibited protective effects on β-cell function and β-cell mass expansion (about approximately 50%-70%) due to β-cell hyperplasia. After two months, the

effects of GLP-1 and exendin-4 treatment persisted as low blood glucose concentration values were similar to the control group (Tourrel et al. 2001). Using the same approach as the previous study, the authors treated neonatal Goto-Kakizaki (GK) rat (obese, spontaneous-genetic diabetes model) with GLP-1 and exendin-4. At postnatal day 7, GK rats treated with either GLP-1 or its agonist had β-cell mass expansion due to an increase proliferation and neogenesis. Lastly, two-month-old GK rats-maintained glucose tolerance and improved β-cell mass in comparison to untreated GK mice (Tourrel et al. 2001).

1.5. Islet cytoarchitecture and function across different species.

Despite extensive research in islet morphology and β-cell function, additional studies are required to understand islet development during early life and the mechanisms that dictate β-cell maturation. Differences in the pattern expression of transcription factors among species exist, yet transgenic mouse models have provided numerous insights into the molecular mechanisms that regulate pancreas development (Jennings et al., 2020). Moreover, rodent models allow an easy accessibility to pancreas tissue at any age and offer approaches to study the effect deleted genes at early life.

The use of pig models has been included in islet development experiments due to similarities to humans in islet physiology and development, especially during the neonatal period (Renner et al., 2020). The following paragraphs will compare neonatal islet development, with specific focus on cytoarchitecture and function among humans, rodent and pigs.

1.5.1. Islet cytoarchitecture. Studies in human islets suggest that development of adult-like islet architecture begins after birth and is fully acquired at 2 years of age (Bakhti et al., 2019; Skelin Klemen et al., 2017). During the transition of early to late infancy, human β-cells can be detected in scattered single cells or organized in small clusters (Gregg et al., 2012). Human

pancreatic β-cells are centered in the core of the islet surrounded by α-cells and δ-cells, whereas newborn mice islets are organized in a cord-like structure with β-cells located in the center and α-cells in the periphery (Steiner et al., 2011). However, by the third postnatal month, human insulin-positive cells are mixed with other pancreatic endocrine cells, adapting an architecture that is more similar to the adult islet (Henquin & Nenquin, 2016; Stefan et al., 1983).

Similar to humans, the architecture of porcine islets is round or oval with insulin-positive cells arranged in small groups or scattered across the pancreatic islet. Porcine glucagon-positive cells are localized within the cores and peripheries of islet clusters (Nagaya et al., 2019; Steiner et al., 2011).

1.5.2. Islet composition and proliferation. Several studies reported that in the first year of life, the β-cell mass changes due to high proliferation that declines in adulthood (Gregg et al., 2012; Henquin & Nenquin, 2016; Nagaya et al., 2019; Skelin Klemen et al., 2017; Smith et al., 2018). During the period after birth and the first 2 years, human pancreatic β-cells proliferation first increases and then gradually decreases until after the age of 3-5 years (Moin & Butler, 2019).

However, in postnatal rodents, a "second wave" of β-cell proliferation has been described in mice before weaning (Bonner-Weir et al., 2016) while in humans and pigs, this "second wave" has not been observed (Bakhti et al., 2019). An increase in β-cell mass that is characterized only in rodents, might be influenced by the context of the species studied. Rodent islets are primarily constituted by β-cells (approximately 80%) and low number of α-cells (<10 %) (Nair & Hebrok, 2015). While in human pancreatic islets are composed predominately of β-cells (approximately 60%), α-cells (approximately 30%) and the remaining 10% consist of somatostatin, PP and ghrelin producing-cells (Moin & Butler, 2019). In neonatal pigs, islets are constituted mainly by β-cells, and the number of α-cells are similar to δ-cells (Kim et al., 2020).

1.5.3. Differences in "β-cell maturity markers". In the neonatal period, β-cell maturation is controlled by the activity of specific transcription factors that are referred as "β-cell maturity markers". Recently, *MAFA* and *MAFB* have been extensively used as such markers because they are under the regulation of *PDX1*; and *MAFA* and *MAFB* are expressed differently among species and vary in an age-dependent manner. *MAFA* and *MAFB* are identified in juvenile α- or β-cells and play a significant role in insulin synthesis, secretion and glucose sensing (Arda et al. 2016; Qiu et al. 2017; Aguayo-Mazzucato et al. 2006). *MAFA* expression is found exclusively enriched in β-cells and increases in an age-dependent manner in humans, pigs and mice (Arda et al. 2016; Jennings et al., 2020; Qiu et al. 2017; Blum et al. 2012; Kim et al. 2020). In human islets, *MAFB* is highly expressed during the fetal period and remains expressed in adult α- and β-cells (Arda et al. 2016), comparable to pigs (Kim et al., 2020). While in newborn rodents, *MAFB* expression decreases gradually and remains undetected in adults (Blum et al., 2012; Cyphert et al., 2019).

Thus, comparative studies regarding islet development have revealed distinct mechanisms of islet cytoarchitecture arrangement and endocrine cell maturation. Validation of techniques used to identify changes in the expression level of key markers related to β-cells should consider the physiology of the animal model used.

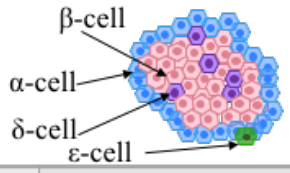
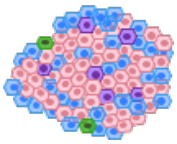
			
Species	**Mouse**	**Pig**	**Human**	**References**
Pancreatic islets characteristics				
Endocrine cells proportion	Primarily constituted by β-cells (65-80%), and low number of α-cells (10-20%) Low number of δ-cells (~5%) and ε-cells (<1%)	Predominately constituted by β-cells and the number of α-cells is similar to δ- and ε-cells.	Predominately constituted by β-cells /50-60%) and α-cells (30-50%). Low number of δ-(~5%) and ε-cells (<1%).	Campbell & Newgard, 2021; Nair & Hebrok, 2015; Moin & Butle, 2019; Kim et al., 2020.
Endocrine cells distribution	α-cells: outer mantle β-cells: inner core	α-cells: within the cores and outer membrane. β-cells: inner core	α-cells: within the core and outer membrane β-cells: inner core	Bakhti et al., 2019; Steiner et al., 2011; Henquin & Nenquin, 2016; Nagaya et al., 2019
Neonatal pancreatic islets				
Proliferation	High –rate of proliferation before weaning "second wave of proliferation"	During the first week after birth	During the first week after birth	Bonner-Weir et al., 2016; Bakhti et al., 2019.
Islets architecture	Islets are organized in a cord-like structure	Scattered single cells or small groups.	Scattered single cells or small clusters, and large islets	Gregg et al. 2012.
Differences in β-cell maturity markers				
Transcription factors	MAFA is enriched in β-cells, MAFB is absent	MAFA is enriched in β-cells, MAFB is enriched in α-cells	MAFA is enriched in β-cells, MAFB is enriched in α-cells	Blum et al. , 2012; Cyphert et al., 2019; Arda et al., 2016; Qiu et al., 2017; Aguayo-Mazzucato et al., 2006.

Table 1.1 Comparison of pancreatic islets function and cytoarchitectures across mouse, pig and human.

1.6. Gut microbiota and type 2 diabetes.

1.6.1. Gut microbiota composition and microbial products. The gut harbors trillions of microbes composed of approximately 200 prevalent bacterial species and up to 1000 unknown or less-common species, that have evolved with the host (Greiner & Bäckhed, 2016). Gut bacteria reside in the colon with an estimated concentration of 10^{11} bacteria per gram of digesta. The bacterial density is much lower in the small intestine, reaching a maximal community of approximately 10^{8} in the ileum; possibly due to higher levels of oxygen, antimicrobial peptides and bile acids (Davenport et al., 2017; Donaldson et al., 2015; Sender et al., 2016). This community of microbes carries a wide number of genes that far surpasses the functional capacity of the human

genome and offers substantial benefits and functions that humans lack without these microbes (Gilbert et al., 2018; Greiner & Bäckhed, 2016).

The gut microbiota generates bioactive nutrients that act as signaling molecules which modulate gene expression in the host, resulting in paracrine and autocrine effects (Priyadarshini et al., 2016). For instance, short-chain fatty acids (SCFA) are bioactive metabolites important for health maintenance. SCFA have a plethora of biological functions in the gastrointestinal tract including the conservation of gut integrity, nutrient absorption, mucus production and the control of inflammation. SCFA also improve energy homeostasis by stimulating insulin secretion, increasing satiety, regulating liver mitochondrial function, and activating brown adipose tissue (He et al., 2020). SCFA are aliphatic compounds that are linear-saturated chains of two to six atoms of carbon. Acetate (C2), propionate (C3), and butyrate (C4) are the major SCFA and are produced largely through the anaerobic fermentation of non-digestible fiber in the cecum and proximal colon; and are found in the large intestine at an approximate molar ratio of 60:20:20 for acetate:propionate:butyrate (Gérard & Vidal, 2019; Venegas et al., 2019). Butyrate is mainly produced by anaerobic gram-positive bacteria like *Roseburia* spp, Ruminococcaceae, *Clostridium leptum*, and *Faecalibacterium prausnitzii* which belong to the Firmicutes phylum. Acetate is produced by Bifidobacteria, *Akkermansia muciniphila*, *Bacteroides*, and *Prevotella*, while propionate can be generated by species belonging to *Bacteroides* and *Roseburia* genera (Feng et al., 2018; Koh et al., 2016).

Butyrate is the main source of energy for colonocytes and is detected at lower concentrations in the gut lumen (20 mM) and blood circulation (1-10 μM). SCFA are absorbed via sodium dependent monocarboxylate transporter-1 (SMCT-1), and monocarboxylate transport 1 (MCT-1) via active transport. The SCFA not metabolized by enterocytes are delivered to the liver

via the hepatic portal venous system (Dalile et al., 2019; He et al., 2020). Most of the propionate is metabolized in the liver, whereas acetate is found in blood circulation at high concentrations up to 150 μM, compared to propionate and butyrate blood concentrations (1-10 μM) (Feng et al., 2018; Gérard & Vidal, 2019).

Acetate, propionate, and butyrate are natural agonists of G-protein couple receptor (GPCR)-43 and GPCR41, also known as free fatty acid receptor (FFAR)-2, and FFAR3 which are expressed in the intestine, pancreas, liver, sympathetic nervous system, adipose and immune tissue, suggesting that SCFA are an important mediator of the crosstalk between gut microbiota and peripherical organs (Gérard & Vidal, 2019; Tolhurst et al., 2012).

SCFA signaling pathways participate in a large number of metabolic functions. Activation of SCFA receptors leads to the production of proglucagon-derive peptides, GLP-1 and GLP-2, which are implicated in glucose and lipid metabolism, insulin secretion, oxidative stress and inflammation (Dalile et al., 2019; Patel et al., 2018). GLP-1 secretion from the pancreas is predominantly known as a modulator of glucose metabolism and its relevant functions have been described earlier in the chapter. GLP-2 is secreted from enteroendocrine L-cells located in the small intestine and colon to promotes nutrient absorption by maintaining gut barrier integrity and morphology (Dalile et al., 2019; Hunt et al., 2021; Tolhurst et al., 2012) . Signaling pathways activated by SCFA, particularly butyrate, influence intestinal epithelial barrier integrity by inducing colonocyte differentiation and proliferation, cell migration and cell membrane assembly, and by stimulating tight-junction proteins, (claudin-1, occludin, and Zonula Occludens-1) gene expression (Feng et al., 2018; Venegas et al., 2019).

The intestinal barrier is a physical barrier constituted by epithelial cells connected through intracellular junctions, elements of the paracellular barrier, that has an important role in nutrient

absorption, secretion of hormones, and protection against pathogen translocation (Buckley & Turner, 2018). The intestinal epithelial cells regulate commensal bacteria tolerance and immunity to pathogens by producing antimicrobial peptides that kill bacteria by inducing permeabilization of the cell, and by inhibiting the activation of Toll-like receptors (TLR)-2 and TLR-4 (Coorens et al., 2017). Moreover, goblet cells secrete mucus to form the mucus layer which is a physical-chemical barrier that contains glycoproteins, like antimicrobial peptides that protect the intestinal epithelial cells from pathogens and toxins (Bahar & Ren, 2013; Mukherjee et al., 2008)

SCFA have been found to promote antimicrobial peptide expression in the lungs, pancreatic islets, and gastrointestinal tissue (Sun & Shang, 2015). In non-obese diabetic (NOD) mice, a model for type 1 diabetes, butyrate has been shown to promote β-cell survival and function by inducing cathelicidins production in the pancreas (Sun et al., 2015).

1.6.2. Gut microbiota composition in type 2 diabetes. While the bacterial community provides many benefits, imbalances of the gut microbiota, also known as dysbiosis, is frequently observed in subjects with type 2 diabetes. Reduced alpha diversity within an individual (represented by richness and evenness of the community) has been associated with the onset of diabetes while the severity of insulin resistance, adiposity, and inflammation is increasing (Chávez-Carbajal et al., 2020; Ghorbani et al., 2021; Ley et al., 2005; Turnbaugh et al., 2006; Zhu & Goodarzi, 2020).

Large-scale projects in populations with different ethnic backgrounds have correlated gut microbiome composition and functionality with traditional clinical biomarkers of type 2 diabetes (Karlsson et al., 2013; Zhang et al., 2013).

The relative abundance of the two main bacterial phyla, Firmicutes and Bacteroidetes, have been associated with insulin resistance and type 2 diabetes, although it has been inconsistent

between studies. In some studies, the abundance of Bacteroidetes was increased in gut microbiota of individuals with type 2 diabetes (Chávez-Carbajal et al., 2020; Liu et al., 2021) while others have found Firmicutes as the dominant phyla (Ahmad et al., 2019; Gaike et al., 2020). Another study found that Firmicutes/Bacteroidetes ratio was significantly higher in patients with type 2 diabetes compared to healthy controls (Karlsson et al., 2013; Larsen et al., 2010; Wang et al., 2012), while another found that this ratio did not differ (Zhang et al., 2013b). The lack of consistency between studies may suggest that the relative abundance shifts at the phylum level is not a determining factor in this disease.

Two large metagenome-wide association studies in Chinese and European population, identified common unique signatures in the gut microbiota of subjects with type 2 diabetes. Different studies have observed low abundances of *Roseburia intestinalis* and *F. prausnitzii* compared to healthy subjects and individuals with prediabetes all (Allin et al., 2015; Karlsson et al., 2013; Pinna et al., 2021; Wang et al., 2012; Zhang et al., 2013b). *Roseburia* and *F. prausnitzii* are known as butyrate-producing bacteria, and their low abundance correlates with increased inflammatory markers and insulin resistance, suggesting that both bacteria have an anti-inflammatory role that contributes to maintaining insulin sensitivity which may help prevent the onset of diabetes (Greiner & Bäckhed, 2011; Sircana et al., 2018; Zhu & Goodarzi, 2020).

Individuals with prediabetes have been found to have an elevated abundance of *Shigella*, *Clostridium*, *Prevotella* and *Lactobacillus* (Karlsson et al., 2013; Pinna et al., 2021; Sato et al., 2014; Wang et al., 2012). Also, different authors reported that *Escherichia* is enriched in both subjects with prediabetes and type 2 diabetes (Gaike et al., 2020; Sato et al., 2014; Wang et al., 2012). Notably, *Escherichia* and *Prevotella* were positively correlated with HOMA-IR in Danish populations with type 2 diabetes (Pedersen et al., 2016). However, Japanese participants with

diabetes had a low abundance of *Prevotella* and did not correlate with clinical parameters (Sato et al., 2014). Elevated abundances of *Prevotella* have related to diabetes incidence due to its role in the production of branch chain amino acids. Furthermore, the increased abundance of Enterobacteriaceae in individuals with type 2 diabetes was positively correlated with proinflammatory cytokine tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), and HbA1c (Sato et al., 2014).

Collectively, these findings highlight the fact that diverse bacterial taxa are associated with altered glucose homeostasis during the progression of diabetes, though few have been demonstrated to have a causal relationship.

1.6.3. Endotoxemia during the onset of type 2 diabetes. High-fat and "Western-style" diets are diets rich in energy calorie content, of which 30-45% of the calories stem from fat intake (Rohr et al., 2020). Prolonged consumption of high-fat diets is linked to dysbiosis and chronic low-grade inflammation observed in type 2 diabetes. This condition is known as endotoxemia which is driven by two mechanisms: the increased production of LPS and intestinal permeability (figure 1.1) (Régnier et al., 2020). The outer cell membrane of Gram-negative bacteria is enriched with LPS, a glycolipid that consist of an O-antigen, core polysaccharide, and lipid A. The structure of lipid A contains branched chain short-fatty acids that are the primary ligands of TLR-4/ LPS-binding protein/CD14 pathway in immune cells (macrophages) and epithelial cells. LPS induce the activation of the cytokines IL-1 and IL-6, which are implicated in metabolic inflammation in peripherical tissues (Cornejo-Pareja et al., 2019). TLR-4 activated by LPS, increases TNF-α gene expression to inhibit the phosphorylation of insulin receptor substate IRS-1, a key component of the insulin signaling pathway (Mandaliya et al., 2021; Matheus et al., 2017). Moreover, TLR-4 and nucleotide oligomerization domain-like receptors in macrophages and dendritic cells,

activated by LPS, activates nuclear factor-ϰB (NF-ϰB) and activator protein 1 (AP-1) gene expression (Boulangé et al., 2016).

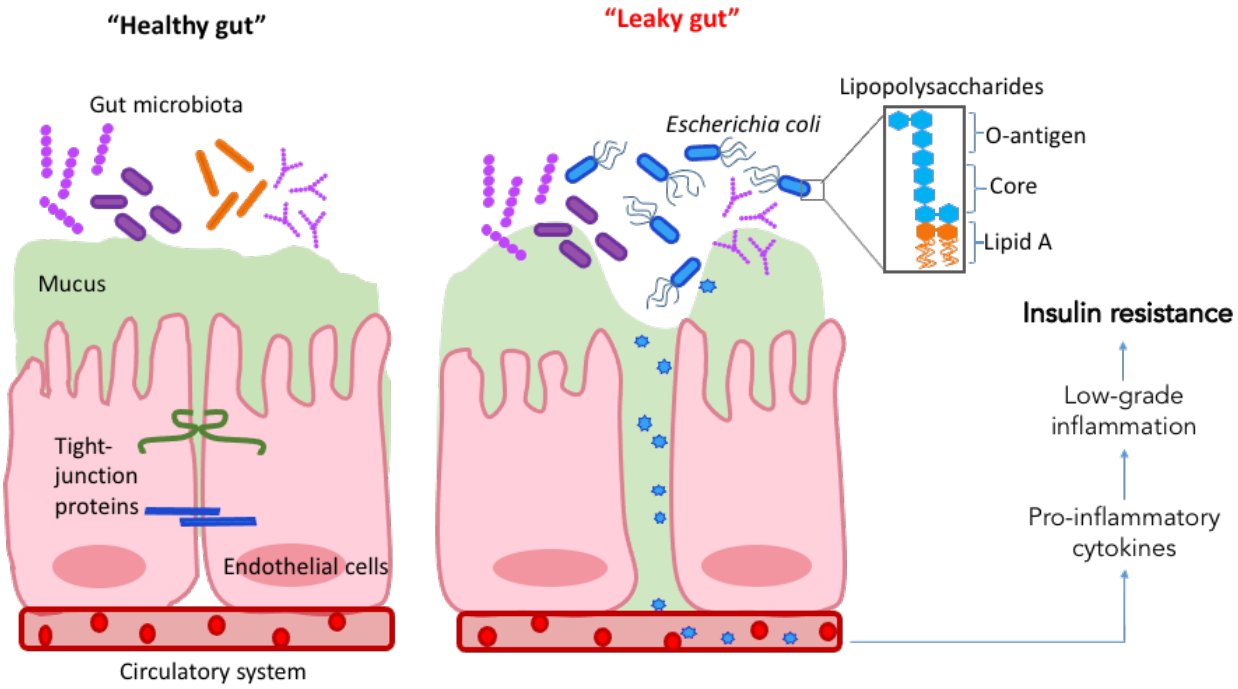


Figure 1.1 Gut barrier alteration during metabolic endotoxemia. In healthy subjects, the optimal gut barrier is composed by chemical (mucus) and physical (epithelial cells) barrier, that are maintained together by tight-junctions proteins. Environmental factors impair gut microbiota composition and gut barrier integrity, facilitating the translocation of microbial products like lipopolysaccharides (LPS) to circulatory system. LPS reach peripherical tissues and initiate a cascade of pro-inflammatory cytokines production that trigger low-grade inflammation that exacerbates insulin resistance in subjects with obesity and prediabetes. Figure obtained and modified from Regnier et al., 2020.

Mouse models of obesity are characterized by a high abundance of Gram-negative bacteria; but some Gram-positive bacteria such as *Eubacterium rectale* and *Clostridium coccoides* have been shown to be increased, while Bifidobacteria are present in lower amounts compared to control groups (Cani et al., 2007). Moreover, this animal model displayed an elevated LPS level and impaired expression of tight-junction proteins, which resulted in increased paracellular intestinal permeability (Caesar et al., 2012; Nascimento et al., 2020). Mice treated with LPS and a high-fat

diet had increased mRNA levels of TNF-α, IL-1, and IL-6, and elevated fasting insulin blood concentration (Cani et al., 2007).

LPS might contribute to early stages of diabetes pathophysiology by inducing changes in proglucagon peptides products, GLP-1 and GLP-2. Nguyen and collaborators (Nguyen et al., 2014) observed that LPS intraperitoneal injection in mice generates a rapid change in glucose homeostasis by a transient but significant hyperglycemia 6 hours after LPS exposure. Moreover, chronic exposure of LPS at low doses enhanced insulin and GLP-1 secretion observed during the OGTT (Nguyen et al., 2014). Lebrun et al (Lebrun et al., 2017) reported that LPS stimulates proglucagon and prohormone convertase 1/3 gene expression in the mouse ileum and induces GLP-1 secretion by the TLR-4 signaling pathway (Lebrun et al., 2017). The selective deletion of the proglucagon gene in the mouse distal gut suggests that major production of LPS-induced GLP-1 originates from the distal gut, and not from other tissues (Panaro et al., 2020).

Maruta et al (Maruta et al., 2020), reported that GLP-2 is released in response to LPS exposure to protect the intestinal paracellular permeability and molecule transport. Mice treated with LPS intraperitoneal injection, exhibited an increase in GLP-2, IL-6, and TNF-α concentration in blood after 6 hrs post exposure. The gut permeability was enhanced compared to the control group, suggesting that GLP-2 maintains gut integrity disrupted by LPS by inducing anti-inflammatory effects (Maruta et al., 2020). Notably, GLP-2 receptor deletion in *ob/ob* mice displayed increased fasting blood glucose and glucagon concentrations, and significantly reduced β–cell mass and islet proliferation, but increased α-cell mass (Bahrami et al., 2010). All together, these findings suggest that GLP-2 prevents metabolic endotoxemia effects by protecting intestinal barrier integrity and regulating mechanisms involved in glucose homeostasis.

Most of the studies showing the implication of LPS in insulin resistance etiology observed that high-fat diet is the major player in the endotoxemia development. Excessive dietary fat consumption increases the synthesis of chylomicrons, which incorporate LPS via lipid A. LPS translocation to the blood can also be facilitated by luminal micellar and lipid-raft mediated endocytosis. These mechanisms are independent of the intestinal permeability status, but mainly enhanced by the consumption of high-fat diets (Rohr et al., 2020).

A study performed by Cani et al (Cani et al., 2008), reported that high-fat diet induced metabolic endotoxemia triggered multiple mechanisms that favored low-grade inflammation. However, the administration of broad-spectrum antibiotics prevented metabolic endotoxemia by inducing changes in gut microbiota composition and functionality which ultimately impacted LPS plasma levels. Mice treated with antibiotics displayed reduced gut permeability, adipose tissue inflammation and glucose tolerance improvement independent of diet (Cani et al., 2008).

Different studies have identified an increase in insulin sensitivity in obese human and mouse models after antibiotic treatment (Hwang et al., 2015; Rajpal et al., 2015; Rodrigues et al., 2017; Vrieze et al., 2014). However, some antibiotics can further stimulate LPS release after bacterial lysis, and enhance immune responses (Sun & Shang, 2015). Jin et al. reported that after penicillin and erythromycin exposure, high levels of circulatory LPS were detected and an increase of genes involved in LPS-induced inflammation were detected in the liver (Jin et al., 2016).

Moreover, an in vitro study reported that LPS released from *Escherichia coli* (*E. coli*) increased due to bacterial growth. *E. coli* was sensitive to the exposure of gentamicin, amoxicillin, and ciprofloxacin, however an increase in LPS levels was observed after antibiotic exposure (Van Den Berg et al., 1992).

These studies suggest that LPS levels can increase due to high-fat diet-induced Gram-negative bacterial growth and antibiotic-induced lysis and affect the activity of various pro-inflammatory cytokines and incretins, causing perturbations in host immune function and metabolism.

1.6.4. Antibiotic-induced perturbations in neonate's gut microbiota. During the first 1000 days of human life, the gut microbiota is characterized by low diversity and high instability and is highly influenced by environmental and host factors (Oldenburg et al., 2018). Mode of delivery, gestational age, and feeding mode are the major determinants of microbial ecology in infants (Milani et al., 2017).

The description of the infant gut microbiota has been challenging due to individual variation stemming from different environmental factors; however, the identification of representative bacteria in healthy term infants have been suggested to be transferred during birth and by breastfeeding (Bokulich et al., 2016; Laforest-Lapointe & Arrieta, 2017). The gut microbiota during this period consists mainly of the phyla Firmicutes and Proteobacteria. Members of the family Enterobacteriaceae, especially *E. coli* are prevalent during this time. Changes in oxygen supply by these facultative anaerobes, such as *E. coli*, allow the colonization of strict anaerobes like Bifidobacteria, *Bacteroides* and *Clostridium* (Derrien et al., 2019; Matamoros et al., 2013). Succession of the gut microbiota during postnatal period is a complex process that involves frequent changes in bacterial species abundance until it resembles an adult-like state at approximately three years of age (Robertson et al., 2019; Schulfer & Blaser, 2015).

Patterns in microbial colonization in the neonatal stage are critical for optimal growth and maturation of the immune system and metabolic organs. Pediatric broad-spectrum antibiotics target a wide number of pathogenic bacteria, but also affect members of the bacterial community

located in the gut, which may confer profound short and long-term effects on the diversity and composition of the gut microbiota (Vangay et al., 2015; Willing et al., 2011)

Infants are in high exposure to antibiotics. One recent study reported that 82.3% of the participants had at least 1 antibiotic course, 30.8% were dispensed at least 5 courses, and 8.4% received 10 or more antibiotic courses within the first two years of life (Leong et al., 2020). Some population-based cohort studies have associated sustained long-lasting effects of antibiotic exposure on metabolism. It has been found that infants exposed to antibiotics during the first 6 months of life were susceptible to an increased body mass index, weight gain, and childhood overweight and obesity, which was adjusted by potential social, family, and biological confounders (Ajslev et al., 2011; Trasande et al., 2013). Moreover, children exposed to a single course of antibiotics during the first six months of life had higher weight z-scores. Administration of β-lactam agents were also highly associated with increased weight z-scores, regardless of the number of antibiotic courses (Mbakwa et al., 2016). Furthermore, Aversa et al reported that antibiotic exposure during the first 2 years of life facilitates a predisposition to obesity with an even higher incidence in girls (Aversa et al., 2021). However, some studies have found no association between childhood obesity and antibiotic exposure after adjustment for family-level confounders (Leong et al., 2020). The limitations of most population-based studies stem from the fact that primary factors contributing to obesity such as maternal body mass index, socioeconomic status, infection itself, host genetics, ethnicity and environmental factors are typically not considered.

A better understanding of the effects of neonatal dysbiosis on metabolism is highlighted in mice models that are exposed to antibiotics in early stages of life. Early-life antibiotic treatment during the weaning period has been associated with negative metabolic outcomes in adulthood (Cho et al., 2012; Mahana et al., 2016; Underwood et al., 2020). Mice treated with antibiotics had

an increased Firmicutes to Bacteria ratio and depleted abundance of *Lactobacillus*. Also, they reported antibiotic induced SCFA elevation associated with changes in liver lipid metabolism that favored increased adiposity (Cho et al., 2012; Nobel et al., 2015)

Similarly, Cox et al (Cox et al., 2014) observed that antibiotic administration pre-weaning combined with a high-fat diet had critical long-term effects that persisted even after antibiotic withdrawal. Moreover, antibiotic administration upregulated hepatic genes involved in lipid metabolism. The incorporation of high-fat diets exacerbated the antibiotic treatment effects, and significantly decreased the expression of glucose metabolism genes in the liver and impaired insulin metabolism. Notably, antibiotic administration during early-life caused the downregulation of antimicrobial peptides β--defensin-1 and regenerating islet-derived protein 3 gamma (RegIIIγ) and reduced IL-17 cells in the ileum. Ultimately, antibiotic exposure induced significant changes in gut microbiota composition which were exacerbated by high-fat diets (Cox et al., 2014).

Another study found that prolonged exposure to subtherapeutic doses of antibiotics starting from the prenatal period, in combination with high-fat diets, enhanced the severity and susceptibility of type 2 diabetes. Mahana and collaborators (Mahana et al., 2016) reported that mice exposed to antibiotics during the prenatal period who were switched to high-fat diets during the eleventh week of life had impaired glucose tolerance, regardless of subtherapeutic doses of antibiotic exposure. However, mice from the antibiotic group displayed insulin resistance, and increased levels of pro-inflammatory cytokines TNF-α and IL-6 in the blood. Mice receiving subtherapeutic doses of antibiotics were found to be enriched with *Bifidobacterium*, S24-7 and *Prevotella* (Mahana et al., 2016).

Other authors have reported that amoxicillin or tylosin exposure during early life increases the susceptibility to severe and prolonged colitis. Antibiotic exposure has been implicated in lower

microbial richness and evenness, and changes in microbial community structure highlighted by an increased abundance of *Clostridium* and Enterobacteriaceae. Furthermore, mice exposed to antibiotics displayed low IL-22, Th17 and Treg cells; TNF-α trended higher, and these mice exhibited greater colonic inflammation, epithelial atrophy and dysplasia (Ozkul et al., 2020).

Overall, the first 3 years of life is a critical window for gut microbiota colonization and maturation. Antibiotic exposure causes shifts in the microbiota during the neonatal period which has been suggested to confer long-term effects on host metabolism and immune function.

Chapter 2. Transient antibiotic-induced changes in the neonatal swine intestinal microbiota impact pancreatic islet expression profiles reducing subsequent function

2.1. Rationale

In humans, the period from preconception to up to 3 years of age is a critical window for optimal development and infants are particularly susceptible to environmental influences during this time (Forgie et al., 2020). After birth, the gut microbial communities undergo gradual succession until they acquire an adult-like structure (Bäckhed, 2011; Bokulich et al., 2016; Forgie et al., 2020). A meta-analysis of 12 studies identified that infants exposed to antibiotics had a relative risk of 1.14 for childhood overweight or obesity (Wan et al., 2020). Newborn mice exposed to penicillin had transiently reduced gut microbial diversity, downregulation of antimicrobial peptides in the ileum and increased body fat at 20 weeks of age. Moreover, the metabolic mechanisms favoring the obese phenotype persisted after antibiotic cessation (Nobel et al., 2015). Since obesity is a major risk factor for type 2 diabetes longer-term follow-up may eventually show increased risk of type 2 diabetes as well.

Previously, we demonstrated that gut microbiota perturbations induced by antibiotics alter development of pancreatic islets and produce longer-term metabolic disturbances (Li et al. 2017). Newborn piglets exposed to therapeutic doses of amoxicillin had a transient shift in gut microbiota composition with a higher abundance of the family Enterobacterioceae at PND7. Islets isolated from antibiotic-treated piglets exhibited elevated insulin content and hyper-responsiveness to glucose at PND21 and mild glucose intolerance was detected at PND49 (Li et al. 2017). At PND21, we found reduced *PDX1* mRNA, reduced apoptotic β-cells and increased insulin content in islets,

but these differences were not observed at PND49 (Li et al. 2017).. However, the persistent impairment of glucose tolerance at PND49 suggested that these early phenotypic changes were important and that microbe-host interactions might be of greater magnitude during the period of antibiotic administration, i.e. between PND0-13.

Insulin synthesis and secretion is tightly regulated by β-cells' capability to sense fluctuations in glucose and other nutrients (Campbell & Newgard, 2021). Epidemiological studies find that stressors applied in utero or neonatally impact β-cell metabolic programming by initially inducing an adaptive response (Nielsen et al., 2014). However, this subsequently increases the risk of β-cell failure during increased metabolic demand, which could predispose to development of diabetes in adulthood (Cohrs et al., 2020; Gregg et al., 2012). Thus, carefully regulated evolution of β-cell functional plasticity in the neonatal period is critical for maintaining optimal glucose metabolism later in life (Aguayo-Mazzucato et al., 2011; Jermendy et al., 2011; Leahy, 2008).

Crucial regulators such as *PDX1* (Puri et al., 2015), *TCF7L2* (Migliorini & Lickert, 2015), *IGF-2* as well as *GLP-1* (Stoffers et al., 2003) and *PSCK1/3* (Arda et al., 2016; Kim et al., 2020) are involved in neonatal β-cell differentiation and maturation. How gut microbial perturbations trigger alterations in these during critical windows of development has not been explored.

The piglet, as an animal model, has similarity to humans regarding gut microbiota composition and function (Burrin et al., 2013) and neonates can be administered antibiotics directly. Moreover, piglet islets undergo developmental stages that are comparable to human infants (Arda et al., 2016; Kim et al., 2020) and the progression of metabolic disorders in pigs is similar to humans (Kim et al., 2020). Hence using pigs rather than rodents will likely provide closer parallels to human islet development.

The aim of the present study is to identify whether antibiotic administration from PND0-13 induces changes in 1) key regulators of pancreatic β-cell mass and function in neonatal piglets, 2) morphology and mass of pancreatic islets cells, 3) circulating LPS and proinflammatory cytokines that might mediate microbe-host interactions, and 4) gut bacteria composition and metabolite production, focusing on PND7 and PND14 during amoxicillin administration as well as PND49, when glucose intolerance was previously observed.

2.2. Materials and methods

2.2.1. Study design, animal husbandry and procedures. The animal study and experimental procedures were approved by the University of Alberta Animal Care and Use Committee (ACUC) and were in accordance with the Guidelines of the Canadian Council on Animal Care. Swine husbandry followed standard operating procedures approved by the Swine Research and Technology Centre, University of Alberta and the ACUC. Reporting follows the ARRIVE Essential 10 guidelines 2.0 (du Sert et al., 2020).

We conducted a longitudinal study of n=42 crossbred piglets (Duroc x Large White/Landrace) obtained from 8 Large White/Landrace sows of parity ≥2. Newborn piglets (PND0) from each litter were assigned to antibiotic (ANTI) or placebo control (CON) groups. Piglets weighing <1 kg were not included. Randomization was blocked by litter and sex and conducted by an animal technician with no connection to the study. Investigators were not blinded to group assignment. Oral amoxicillin (30 mg/kg/day) or placebo were administrated twice daily (0800h, 1600h) from PND0-13. Swine were maintained on a 12-h light-dark cycle at room temperature of 22-25ºC with free access to water, food or sow's milk. Body weights were recorded weekly. Piglets at PND7 (n=7) and PND14 (n=7) were euthanized by captive bolt for tissue

collection. The remainder (n=7 per group) were weaned at PND21 and penned by litter in a nursery room with a standard phase-feeding program until euthanized at PND49.

2.2.2. Primary and secondary outcomes. The primary outcomes at PND7, 14 and 49 were non-fasted plasma glucose and insulin, islet insulin content, glucose-stimulated insulin secretion, and β- and β-cell mass. Secondary outcomes included body weight, indices of islet cell proliferation and apoptosis and mRNA expression of genes important to β-cell functional maturation and expansion. Expression of genes or proteins involved in pro- and anti-inflammatory responses was probed. The gut microbiome was characterized at PND7 along with selected metabolites of interest.

2.2.3. Materials. Experimental materials were from Millipore Sigma Canada Ltd. (Oakville, ON, Canada) unless otherwise indicated.

2.2.4. Fecal swabs, blood, and tissue collection. Blood samples were aseptically collected from the jugular vein for LPS and insulin analysis. These plus the ileum tissue and contents (snap-frozen in liquid nitrogen) were stored at -80ºC. Pancreas from adjacent the spleen was sectioned, weighed and retained in ice-cold Hank´s Balanced Salt Solution (HBSS; supplemented with 0.2% bovine serum albumin (BSA), 25mM HEPES, 100mg/L L-glutamine and 0.35g/L NaHCO3) for 3-4h prior to islet isolation. A second sample was fixed in 10% phosphate-buffered formalin for 24h for immunofluorescence assays.

2.2.5. Pancreatic islet isolation and insulin secretion assay. Islets were obtained from pancreas using a published protocol (J.Li et al. 2017) then overnight cultured in supplemented CMRL1066 medium (0.5% BSA) at 37°C in humidified air containing 5% CO_2 (Li J. et al.2017) Islets were washed with Dulbecco´s modified Eagle's medium (DMEM; Gibco, Burlington, ON, Canada) containing 0.1% BSA. Quadruplicate samples of 3 islets/vial were incubated in 2.8- or

22-mM glucose in DMEM for 90 min at 37ºC and 5% CO_2. Supernatant fractions were retained and the remaining insulin in islets was extracted with 3% acetic acid and frozen at -20°C for radioimmunoassay. The amount of insulin secreted into supernatant and the amount of insulin remaining in the islet pellet were added to calculate the total islet insulin content. To normalize for islet size the percentage of total insulin secreted was calculated. The ratio of insulin release in response to high (22 mM) versus low (2.8 mM) glucose concentration was used to calculate the insulin release index.

2.2.6. Immunofluorescence studies. Pancreas sections (5μm) on glass slides were blocked with goat serum (30 min, room temperature) followed by incubation (4ºC, overnight) with guinea pig anti-insulin (dilution 1:200) (Dako, Burlington, ON, Canada), mouse anti-glucagon (1:250) and rabbit anti-Ki67 (1:250) (Abcam, Toronto ON, Canada). After washing, slides were incubated (2h, room temperature) with an appropriate secondary antibody (1:500): goat anti-guinea pig (IgG H&L, Invitrogen, Burlington, ON, Canada), goat anti-rabbit (IgG H&L, Invitrogen) or goat anti-mouse (IgG H&L, ThermoFisher Scientific). After washing, sections were mounted with Prolong Gold Antifade Mountant with DAPI (Invitrogen). TUNEL staining was performed using In Situ Cell Death Detection Kit following the manufacturer's instructions to identify apoptotic cells.

Immunofluorescent-stained slides were tile-imaged and stitched at 10X magnification using a Zeiss Colibri microscope. Using ImageJ (http:///rsb.info.nih.gov/ij), images of entire pancreatic tissue sections were batch-processed and analyzed to characterize pancreatic islet morphology and development. A binary mask generated from DAPI staining was used to colocalize the nuclear stains (Ki67, TUNEL) to the nucleus to exclude non-specific (e.g. cytosolic) staining. The structures captured by this analysis were further filtered by size and circularity such that small and/or aberrant non-specifically stained areas were avoided.

Clustering of pancreatic endocrine cells was measured as a sub-analysis of α- and β-cell area in Image J. Stained cells less than 6-pixels apart were considered part of the same cluster entity. Each stained entity, containing one or more α- or β-cells, was categorized based on their total stained area as a non-cluster (165-799 pixels^2, i.e. singlets, doublets and triplets), small cluster (800-2500 pixels^2), or large cluster (>2500 pixels^2).

2.2.7. Quantitative real-time PCR. Total RNA from pancreas, ileum, and proximal colon was extracted using the GeneJET RNA Purification Kit (ThermoFisher Scientific). DNase (RNase-free DNase set; Qiagen, Valencia, CA) was applied on the column to eliminate genomic DNA contamination. Extracted RNA purity and concentration was determined with a Nano Drop 2000c. cDNA was synthesized using Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific). Gene expression was measured by RT-qPCR using PerfeCTa SYBR Green SuperMix Rox (Quanta Bioscience Inc., Gaithersburg, MD) on a StepOnePlus Real-time PCR System equipped with StepOne software v2.3 (Applied Biosystems, ON, Canada). The primers were designed in Beacon Designer 7.9 using the sequences obtained from the ENSEMBL pig database (Supplementary Table 1). The qPCR protocol was: 3 min at 95ºC, 40 cycles of 10s at 95ºC, 30s at annealing temperature. All samples were measured in duplicate and the values were normalized relative to glyceraldehyde phosphate dehydrogenase (Gapdh) using the comparative CT ($2^{-\Delta\Delta CT}$) method. The amplification specificity was verified by melt curve analysis and evaluation of efficiency (90-110%). A no-reverse transcriptase control was used as negative control for DNA contamination.

2.2.8 Western blot. Frozen samples were thawed on ice and homogenized in RIPA buffer. Total protein was measured using Lowry protein assay. Samples (200 µg protein) were subjected to SDS-PAGE, then blotted onto nitrocellulose membranes. The membranes were blocked in 3% BSA/1% Tween/PBS (60 min), then incubation with primary antibodies for tight junction proteins

(Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in TBS/1% Tween/0.5% BSA (4°C, overnight). Membranes were subsequently incubated with appropriate peroxidase-conjugated secondary antibodies (2h, room temperature). ECL Plus (ThermoFisher Scientific) was applied to the membranes and digital images captured. Labeling of specific proteins was compared with GAPDH (used as loading control) using ImageJ.

2.2.9. Serum LPS and hormone assays. Serum LPS was measured by PYROGENT-5000 Kinetic Turbidimetric LAL Assay (Lonza, Burlington, ON, Canada). Plasma insulin was measured by ELISA (Alpco Diagnostics, Salem, NH, USA). Total active GLP-1 was measured using V-PLEX Total kit (Meso Scale Discovery, Rockville, MD, USA).

2.2.10. SCFA measurement. Samples of distal colon contents were prepared and measured as described (J.Li et al.2017) Gas chromatograph peaks were analyzed using Galaxie Software (Varian Inc., Palo Alto, CA, USA) and concentrations of SCFA were calculated. The sum of detected SCFA concentrations represent the total SCFA concentration and the results were normalized per gram of sample (J. Li et al., 2017)

2.2.11. Ileal DNA extraction and 16S rRNA gene amplicon sequencing. Genomic DNA was extracted from frozen ileal digesta samples as previously (J. Li et al., 2017) and quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit (ThermoFisher Scientific). DNA (5 ng/µl) was amplified using universal primers targeting the V3 to V4 region of the bacterial 16S rRNA gene using KAPA HiFidelity Hot Start Polymerase (Kapa Biosystems Inc., Wilmington, MA, USA) and subsequent library preparation was performed according to Illumina's 16S Metagenomic library preparation guide. The final pooled library was diluted to 8 pM in Illumina HT1 buffer and spiked with 10% PhiX control before loading. A MiSeq 600 cycle V3 kit was used for sequencing.

Sequence data were analyzed using a QIIME2 pipeline (Qiime2 v2019.4) (Bolyen et al., 2019). Sequences were checked for quality and trimmed if the average quality was <20, resulting in the forward and reverse reads being truncated at 260 and 220 nt, respectively. The Dada2-plugin was used to denoise the sequence data and generate feature data tables (Callahan et al., 2016). The amplicon sequence variants were aligned with mafft (Katoh et al., 2002) to construct a phylogenetic tree with fasttree2 (Price et al., 2010). Taxonomic classification of the bacterial 16S rRNA gene sequences were made using the Greengenes (13_8 release) reference sequence database (McDonald et al., 2012), with amplicons for the domain of interested extracted using the primer sequences targeting the V3 and V4 regions of the 16S rRNA gene and the q2-feature-classifier extract_read method (Bokulich et al., 2018).

Data was rarefied to 11000 reads per sample and the R package, Phyloseq, was used to visualize changes to microbial community diversity using Chao1, Shannon, and Simpson indices and microbial community structure using the Bray Curtis dissimilarity and principal-coordinate analysis (PCoA) with the ADONIS statistic used to test differences between treatment groups (McMurdie & Holmes, 2013). Differential abundance of predominating taxa presents in >50% of samples at > 0.1% abundance at the genus level were compared between ANTI and CON treated pigs using a Wald parametric test in DESeq2 Bioconductor package in R using a false-discovery rate (FDR) threshold of 0.15 (Love et al., 2014)

2.2.12. Statistical analysis. Kolmogorov-Smirnov test was used to establish normal distributions. Comparisons between groups were analyzed by Student's t-test or two-way ANOVA (or equivalent for non-normally distributed data) followed by Bonferroni post-hoc test for pairwise comparisons. Statistical analysis was performed using GraphPad Prism v6.02 (La Jolla, CA, USA).

Data were expressed as mean ± standard error of the mean (SEM) and results were considered statistically significant at $p<0.05$. P values between 0.1 and 0.05 were considered to be trends.

Name *	**Accession Number**	**Forward 5'-3'**	**Reverse 5'-3'**
Pdx1	NM_001141984.1	TCCCGTGGATGAAGTCTACC	TTGTCCTCCTCCTTTTTCCA
Ins	NM_001109772.1	CAAGCAGGTCCTCACCCC	CACACCAGGTACAGCGCC
Pcsk1	NM_214038	TGGAAGCAAATCCAAATCTC	CCGACTGTTCACCATCAAGC
Pcsk2	NM_001004044.1	ACAAGTGGCCTTTCATGACC	CTCTTCCTCCAGCTCCTCCT
Dpp4	NM_214257.1	CAGGCCCTTGTAGTCAAAAA	GGTAACCACTTCCTCTGCCA
Glp1r	NM_001256594.1	TACTTCTGGCTGCTGGTGGAG	ACCCCAGCCTATGCTCAGGTA
Tcf7l1	XM_005657489.3	CACATGGTCCCTCCGCATCA	CACTCTGCGAGGATTCCTGC
Gapdh	NM_001206359.1	GTTTGTGATGGGCGTGAAC	ATGGACCGTGGTCATGAGT
Gcg	NM_214324.1	CAAGAGGAACAAGAATAACAT	AAGAACTTACATCACTGGTA
Tnf	NM_214022.1	CCAATGGCAGAGTGGGTATG	TGAAGAGGACCTGGGAGTAG
Igf2	NM_213883.2	AGGGCATCCAAACCACAAAC	GGGTTCAATTTTTGGTATGTAACTTG
Il6	NM_214399.1	ATCAGGAGACCTGCTTGATG	TGGTGGCTTTGTCTGGATTC
Npg1	NM_001123149.1	TGGATCAGATCAAGGACC	ACACAGACGCAGAACCTAC

Table 2.1. Primer sequences used in the qPCR for swine mRNA

*Gene names: *Pdx1,* pancreatic and duodenal homeobox 1; *Ins*, insulin; *Pcsk1,* proprotein convertase subtilisin/kexin type 1; *Pcsk2, proprotein convertase subtilisin/kexin type 2; dpp4,* dipeptidyl peptidase 4; *Glp1r,* glucagon like peptide 1 receptor; *Tcf7l2,* transcription factor 7 like 2; *Gapdh,* glyceraldehyde-3-phosphate dehydrogenase; *Gcg,* glucagon; *Tnf,* tumor necrosis factor (alpha); *Igf2,* insulin like growth factor 2; *Il6*, interleukin 6; *Npg1,* protegrin 1

2.3. Results

2.3.1. Metabolic phenotype of piglets. Previously, amoxicillin administered to piglets from PND0-13 resulted in increased β-cell mass at PND21 and mild glucose intolerance at PND49 (Li et al., 2017). We repeated the neonatal antibiotic treatment experiment to examine phenotypic changes present at PND7 and 14. Follow-up at PND49 was also carried out for the present study. Body weight (Figure 2.3.1. A) and blood glucose concentration (not fasted) (Figure 2.3.1. B) were not different between treatment groups. Plasma insulin varied with age ($p<0.02$) and a trend to age x treatment interaction ($p=0.056$) that was most evident at PND49, when ANTI exhibited a 2-fold increase in plasma insulin relative to PND49 CON (Figure 2.3.1. C). Islet insulin content increased with age ($p<0.05$) and a treatment effect was detected in PND49 islets (Figure 2.3.1. D) with ANTI having lower content compared with CON ($p<0.05$). A trend ($p=0.071$) for elevated insulin release index in ANTI pigs was noted at PND7 and 14 but was not sustained at PND49 (Figure 2.3.1. E).

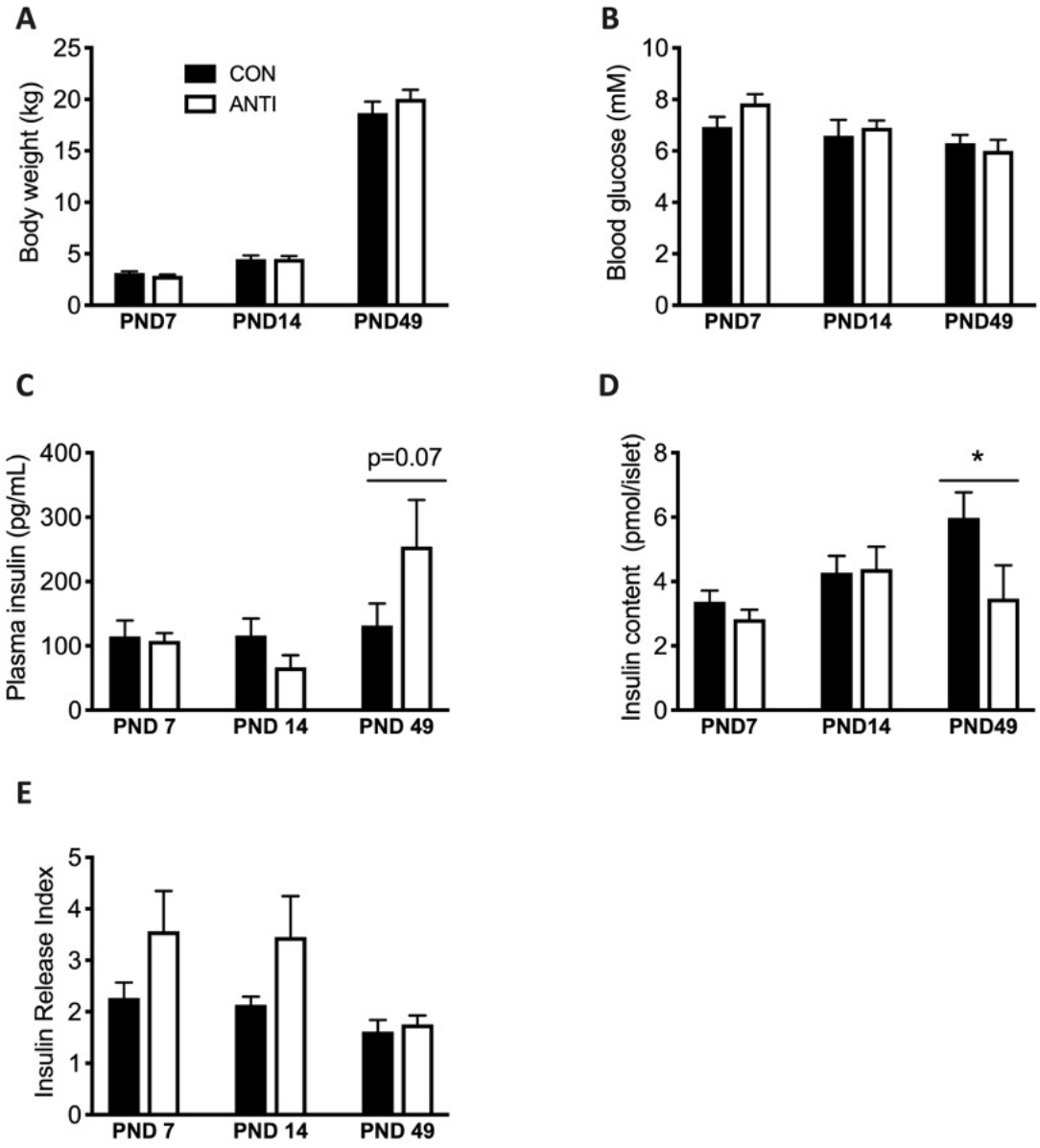


Figure 2.3.1. Body weight, circulating glucose and insulin concentrations, and islet insulin characteristics. **(A)** Body weight, **(B)** random (non-fasted) blood glucose concentration, and **(C)** random plasma insulin concentration of pigs exposed to antibiotics or placebo at PND 7, 14 and 49. **(D)** Pancreatic islet insulin content and **(E)** insulin release index of PND7, 14 and 49 islets from ANTI and CON piglets. ANTI – white bars; CON – black bars. Results are expressed as means ± SEM with n=4-7 per treatment group; *p <0.05 vs age-matched controls.

2.3.2 Immunohistochemical studies of pancreatic islets at PND 7 and 49. Pancreas tissues stained for Ki67, a nuclear marker for cell proliferation nuclei were quantified within cells staining for glucagon or insulin (Figure 2.3.2. A) at PND7. Similar experiments were performed with TUNEL assay (Figure 2.3.2. B). No significant treatment effect on the proportion of co-localized Ki67-positive nuclei (Figure 2.3.2.C,D) or TUNEL-positive nuclei (Figure 2.3.2. E,F) were observed in either α- or β-cells. To explore selected pathways involved in β-cell development and functional maturation, we measured key markers of β-cell maturity (Salinno et al., 2019; Takamoto et al., 2014) We detected a transient reduction in gene expression of *PDX1, IGF-2*, and *TCF7Ll2* (Figure 2.3.2. G-J) in ANTI islets at PND14. The expression of *Ins* mRNA was not different between treatment groups at any age (Figure 2.3.2. I).

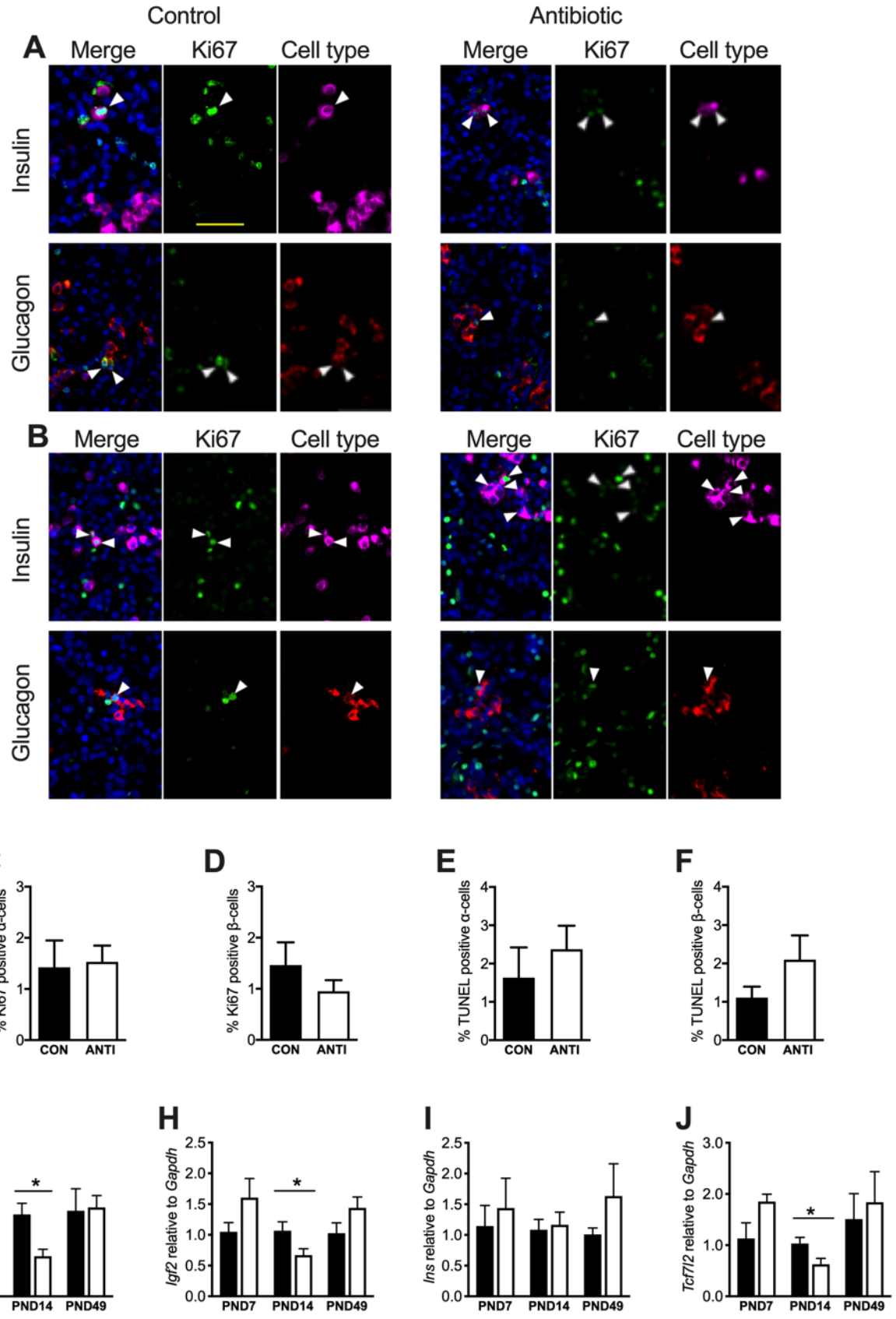

Control
Antibiotic
A
Merge
Ki67
Cell type
Insulin
Glucagon
B
C
% Ki67 positive α-cells
D
% Ki67 positive β-cells
E
% TUNEL positive α-cells
F
% TUNEL positive β-cells
CON
ANTI
G
Pdx1 relative to Gapdh
H
Igf2 relative to Gapdh
I
Ins relative to Gapdh
J
Tcf7l2 relative to Gapdh
PND7
PND14
PND49
*

Figure 2.3.2. Proliferation and apoptosis in α- and β-cells in PND7 piglets and expression of selected genes important in β-cell development and maturation in PND7, 14 and 21 piglets. Pancreatic tissues slices were co-stained with Ki67 (proliferation marker) or TUNEL (apoptosis assay) and glucagon or insulin antibodies. For CON (left panel) and ANTI, representative images of **(A)** Ki67 (green) and glucagon (purple, top) or insulin (red, bottom) and **(B)** TUNEL (green) and glucagon (purple, top) or insulin (red, bottom) are depicted. White arrowheads indicate the nuclei of individual cells that co-stain as seen in the merged images. The yellow bar represents 50 μm. Bar graphs representing the average % Ki67-positive and TUNEL-positive for **(C,E)** α-cells and **(D,F)** β-cells. Gene expression of **(G)** *Pdx1*, **(H)** *Igf2*, **(I)** *Ins*, and **(J)** *Tcf7l2* in isolated islets from CON (black bars) and ANTI (white bars) at PND7, 14 and 49 pigs. Results are expressed as means ± SEM with n=6-7 per treatment group; *p <0.05 vs age-matched controls.

2.3.3. Pancreatic islets morphology of PND7 and PND49. To characterize the abundance of endocrine cells in the pancreas of ANTI versus CON piglets, we measured α-cell and β-cell areas at PND7. The % pancreatic area occupied by α-cells and β-cells (Figure 2.3.3. A, C, D) was similar in ANTI and CON. During development, endocrine cell morphology changes from individual cells or small clusters to larger clusters (Figure 2.3.3. B) that become identifiable as highly organized islet structures around PND21 (Nagaya et al., 2019). Cluster density was not significantly different for small or large clusters of β-cells (Figure 2.3.3. E, F) between the CON and ANTI piglets at PND7. Similar analyses were conducted for α-cells and no significant differences were detected. At PND49, ANTI pigs had a 70% reduction in α-cell area (p=0.02), and a 40% reduction in β-cell area (p=0.005) compared with CON (Figure 2.3.3. G-I).

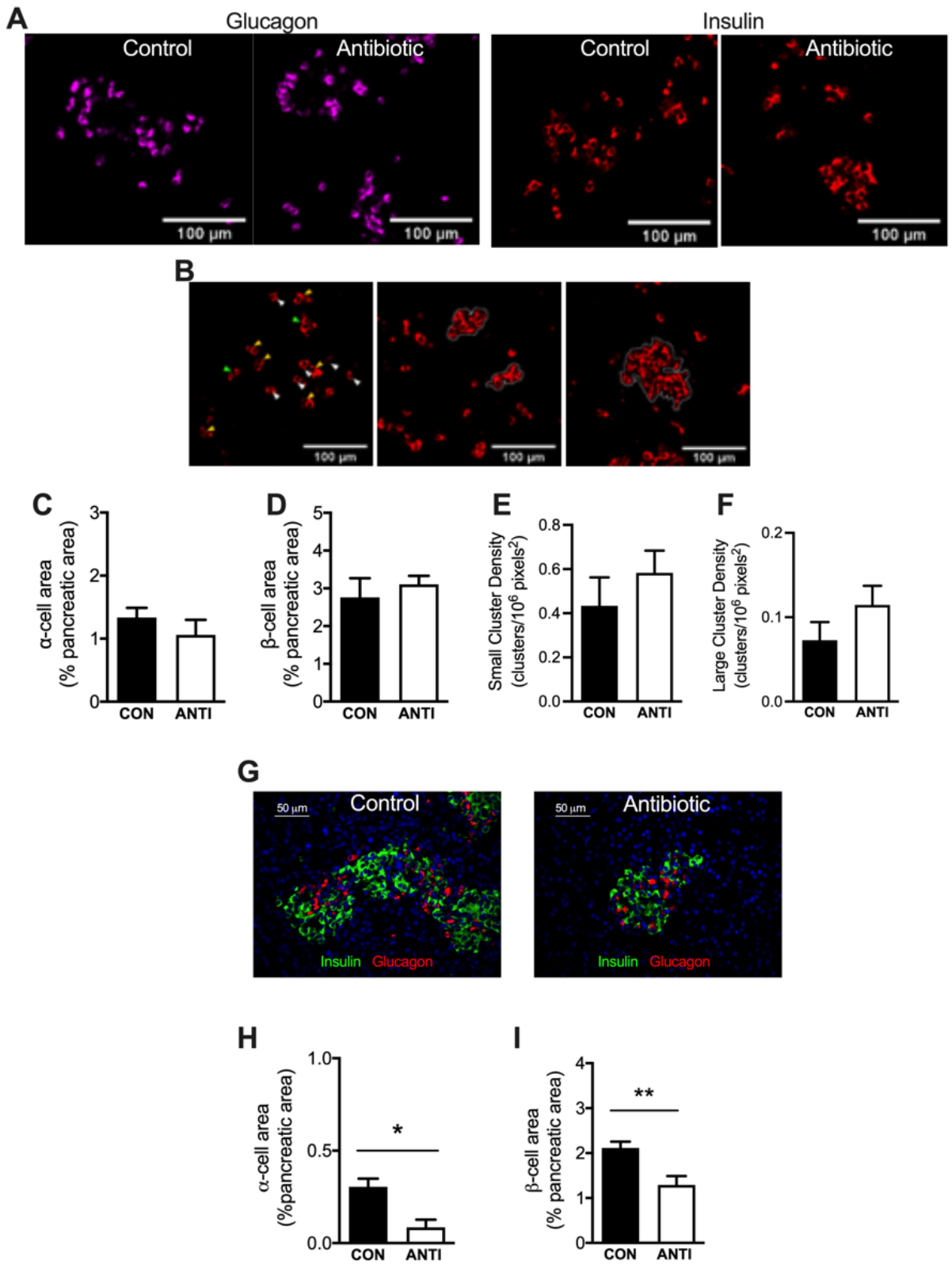

A
Glucagon
Control
Antibiotic
Insulin
Control
Antibiotic
100 µm
B
C
α-cell area
(% pancreatic area)
CON
ANTI
D
β-cell area
(% pancreatic area)
E
Small Cluster Density
(clusters/10^6 pixels^2)
F
Large Cluster Density
(clusters/10^6 pixels^2)
G
50 µm
Control
Antibiotic
Insulin Glucagon
H
α-cell area
(%pancreatic area)
*
I
β-cell area
(% pancreatic area)
**

Figure 2.3.3. Effects of neonatal amoxicillin treatment on islet morphology in PND7 and 49 piglets. (A) Representative immunofluorescent images of glucagon-positive α-cell (left) and insulin-positive β-cell staining (right) at PND7. **(B)** Representative images to demonstrate (left) non-clustered (165-799 pixels^2, white arrows point to singlets, yellow arrows point to doublets, green arrows point to triplets), (middle) small clusters (800-2500pixels^2, white perimeter outlines the computer-generated region of interest identifying the cluster) and (right) large clusters (right) of β-cells (>2500pixels^2, white perimeter outlines the computer-generated region of interest identifying the cluster). The small or large cluster distribution density was quantified by dividing the number of clusters by the total area analyzed for each sample. Morphometric analysis of **(C)** α-cell area, **(D)** β-cell area, **(E)** small clusters of β-cells, **(F)** large clusters of β-cells at PND7. **(G)** Representative immunofluorescent images of glucagon-positive α-cell and insulin-positive β-cell staining in CON (left) and ANTI (right) at PND49. Morphometric analysis of **(H)** α-cell area and **(I)** β-cell area at PND49. CON - black bars and ANTI - white bars. Results are expressed as means ± SEM with n=7 per treatment group; *p <0.05, **p<0.01 vs age-matched controls.

2.3.4. Glucagon-like peptide-1-related genes in PND7 islets. GLP-1 may influence β-cell insulin secretion and mitogenesis via TCF7L2-related signaling pathways (Mitchell et al., 2015). We compared GLP-1 precursor gene *Gcg*, and *Glp-1R* and genes of enzymes involved in GLP-1 synthesis in isolated islets and found significant interactions between age and treatment. GLP-1R ($p<0.05$) and *Gcg* ($p<0.01$) expression were both increased approximately 3-fold in ANTI pigs at PND7 (Figure 2.3.4. A, B). *Psck1* and *Psck2* were also elevated ($p<0.01$) at PND7 (Figure 2.3.4. C, D). For *Dpp4* mRNA was increased in PND7 ANTI pigs but declined thereafter, whereas its expression was relatively constant in CON piglets (Figure 2.3.4. E). Age had a significant main effect on the circulating GLP-1 concentration ($p<0.05$). At PND7, but not later ages, there was a 2-fold elevation ($p=0.11$) of GLP-1 in the plasma of ANTI piglets (Figure 2.3.4. F). Gcg is also expressed in the enteroendocrine L-cells from ileum and colon (Lebrun et al., 2017; Nguyen et al., 2014). Amoxicillin did not affect *Gcg*, *Psck1*, *Psck2*, and *Dpp4* mRNA expression at PND7 (Figure 2.3.4. G), or PND14 and D49 (not shown) in the ileum of CON versus ANTI pigs.

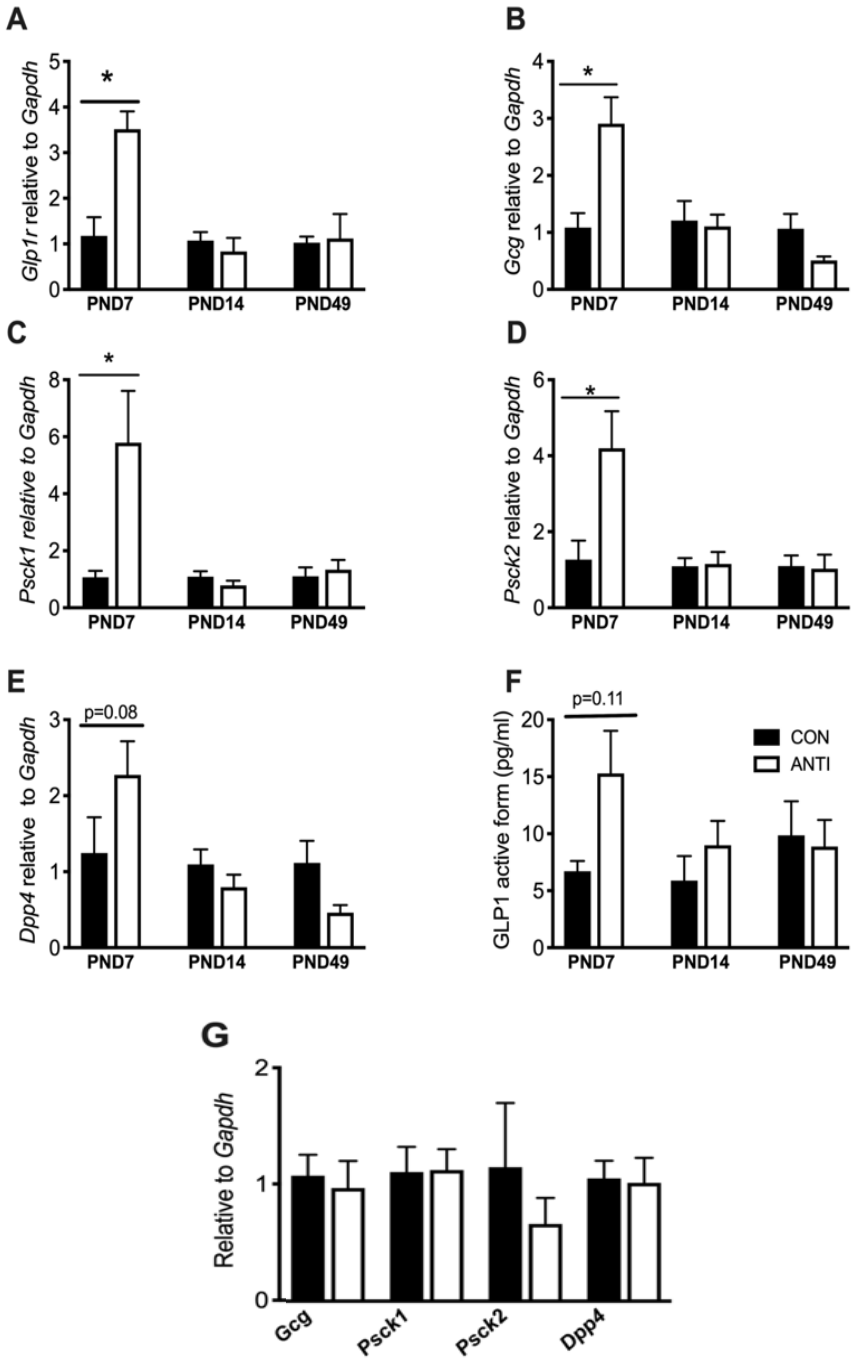


Figure 2.3.4. Plasma GLP-1 and gene expression of pancreatic GLP-1 receptor and enzymes involved in GLP-1 synthesis in islet cells and mRNA expression of **(A)** *Glp1r*, **(B)** *Glc*, **(C)** *Psck1*, **(D)** *Psck2*, and **(E)** *Dpp4*, in pancreatic islet-like clusters of pigs at PND 7, 14 and 49. **(F)** Circulating active GLP-1 and **(G)** measurement of mRNA levels of the same GLP-1-related genes in whole ileum at PND7. Results are expressed as means ± SEM with n=7 per treatment group; *p <0.05 vs age-matched controls.

2.3.5. Peptides associated with inflammation in PND7 ANTI piglets. To address whether amoxicillin administration during early life promoted inflammatory responses, *Tn*f and *Il6* gene expression were measured in pancreatic islets and ileum of PND7 pigs. We observed a significant interaction between age and treatment on *Tnf* expression ($p<0.05$) in pancreatic islets (Figure 2.3.5. A). In the ileum of ANTI pigs, *Tnf* was decreased at PND14 ($p<0.05$) but increased at PND49 relative to CON ($p<0.01$) (Figure 2.3.5 B). Il6 expression in pancreatic islets and ileum were similar in ANTI and CON pig tissues at all ages (Figure 2.3.5 C,D).

Protegrin (*Npg1*) is a porcine cathelicidin that is detected neonatally but not in adulthood and has antimicrobial and anti-inflammatory activity (Hua Wu et al., 1999). *Npg1* mRNA in pancreatic islets was 30-fold higher in ANTI pigs than CON at PND7 ($p<0.05$) but this difference was not maintained at PND14 and PND49 (Figure 2.3.5. E,F).

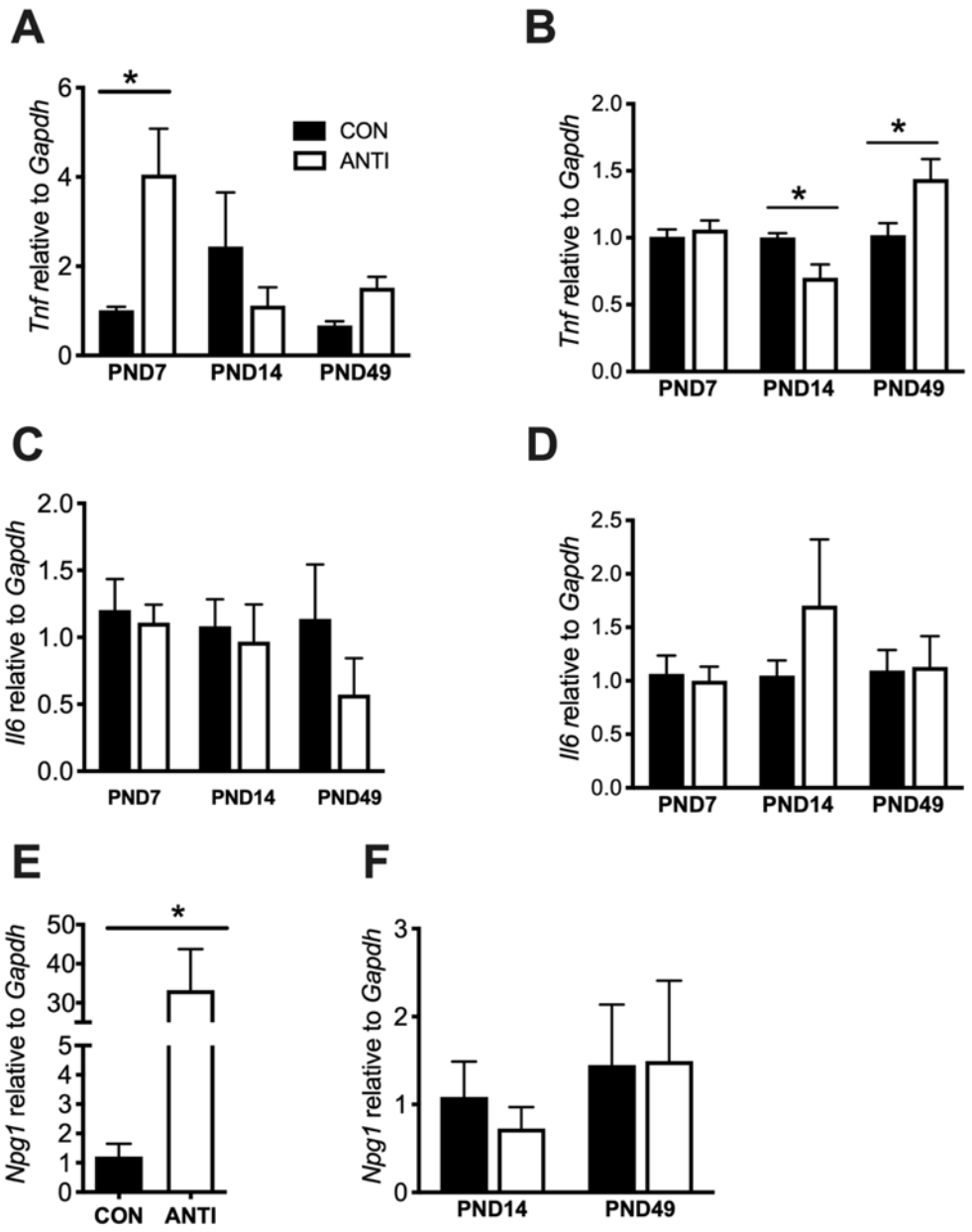


Figure 2.3.5. Measurement of *Tnf*, *Il6* and *Npg1* gene expression in pancreatic islets and ileum of PND7 pigs. Gene expression of *Tnf* in **(A)** pancreatic islets and **(B)** ileum. Il6 mRNA in **(C)** isolated islets or **(D)** ileum. *Npg1* gene expression in pancreatic islets at **(E)** PND7, **(F)** PND14 and PND49. n=3-7 per treatment group. Results are expressed as means ± SEM. * $p<0.05$ vs controls.

2.3.6. Short-chain fatty acids receptors and concentration in PND7 ANTI piglets. The activation of G-protein coupled receptor-41 (GPR41) and GPR43 by SCFA may stimulate cathelicidin production under some conditions (J. Sun et al., 2015). The concentrations of total SCFA (Figure 2.3.6. A), acetate (Figure 2.3.6. B), propionate (Figure 2.3.6 C) and butyrate (Figure 2.3.6. D) were not different between the ANTI and CON piglets at PND7. No differences were detected in the gene expression of *Gpcr41* and *Gpcr43* in ileum (Figure 2.3.6 E) or pancreatic islets (Figure 2.3.6. F) between CON and ANTI piglets

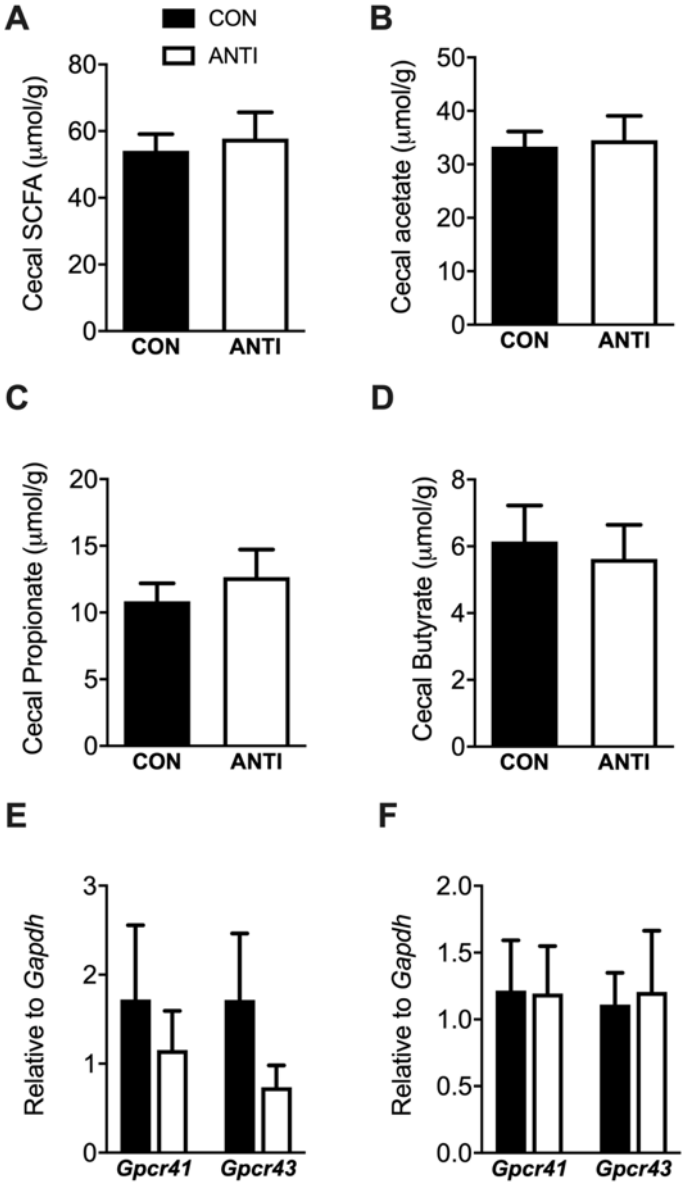


Figure 2.3.6. Analysis of SCFA concentration and receptor expression in PND7 piglets. (A) Total SCFA, **(B)** acetate **(C)** propionate, **(D)** and butyrate concentrations in cecal samples. mRNA expression of SCFA transporters in **(E)** ileum and **(F)** pancreatic islets. n=5-7. Results are expressed by means ± SEM for CON (black bars) and ANTI (white bars). No significant differences were detected.

2.3.7. Neonatal antibiotics transiently alter tight junction protein abundance. We explored whether antibiotic treatment could lead to changes in the mucosal barrier in the ileum during postnatal development because of the increased expression of pro-inflammatory genes. There was a significant treatment x age interaction for claudin-1 ($p=0.02$), ZO-1 ($p<0.05$) and occludin ($p<0.05$). Notably, the abundance of tight junction proteins was lower in ANTI than CON at PND7, but the trend reversed at PND14 and by PND49 there was no difference between groups (Figure 2.3.7.).

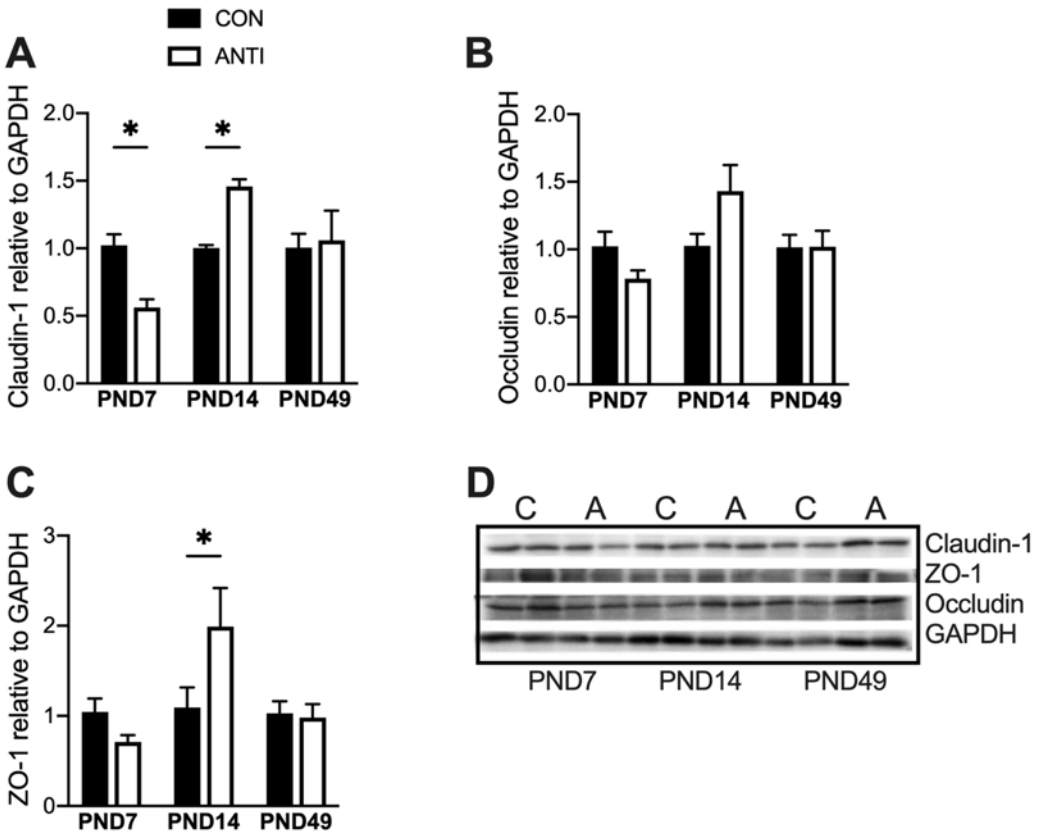


Figure 2.3.7. Tight junction proteins in ileum at PND 7, 14 and 49. (A) Claudin-1, **(B)** Occludin and **(C)** ZO-1. **(D)** shows a representative blot for CON (C) and ANTI (A) at each age. n=6 per group. Results are expressed by means ± SEM for CON (black bars) and ANTI (white bars). *p<0.05.

2.3.8. Ileal microbial composition is transiently altered in ANTI piglets at PND7.

Amoxicillin treatment elicited a pattern of increased Enterobacteriaceae and modest changes in the overall community at PND21 (Li et al., 2017; Nagaya et al., 2019) Ileal microbial diversity and composition was measured at PND7. Species richness was increased in ANTI pigs (Chao1 p=0.005, Figure 2.3.8.A), but other measures of alpha-diversity were not different. PCoA indicated that the ileal microbial community structure was also significantly affected by amoxicillin treatment at PND7 (Adonis p=0.026, Figure 2.3.8 B) that was mostly due to enriched taxa in ANTI piglets in families Ruminococcaceae, Lachnospiraceae, and S24-7 along with genera *Escherichia*, *Coprococcus*, *Ruminococcus*, *Dhalobacterium*, *Oscillospira*, and candidate genus *Ruminococcus* (Figure 2.3.8 C). Circulating LPS (Figure 2.3.8. D) was measured because of the previously observed increase in Enterobacteriaceae, (Fouhse et al., 2019)and was 7-fold higher in ANTI (p=0.11).

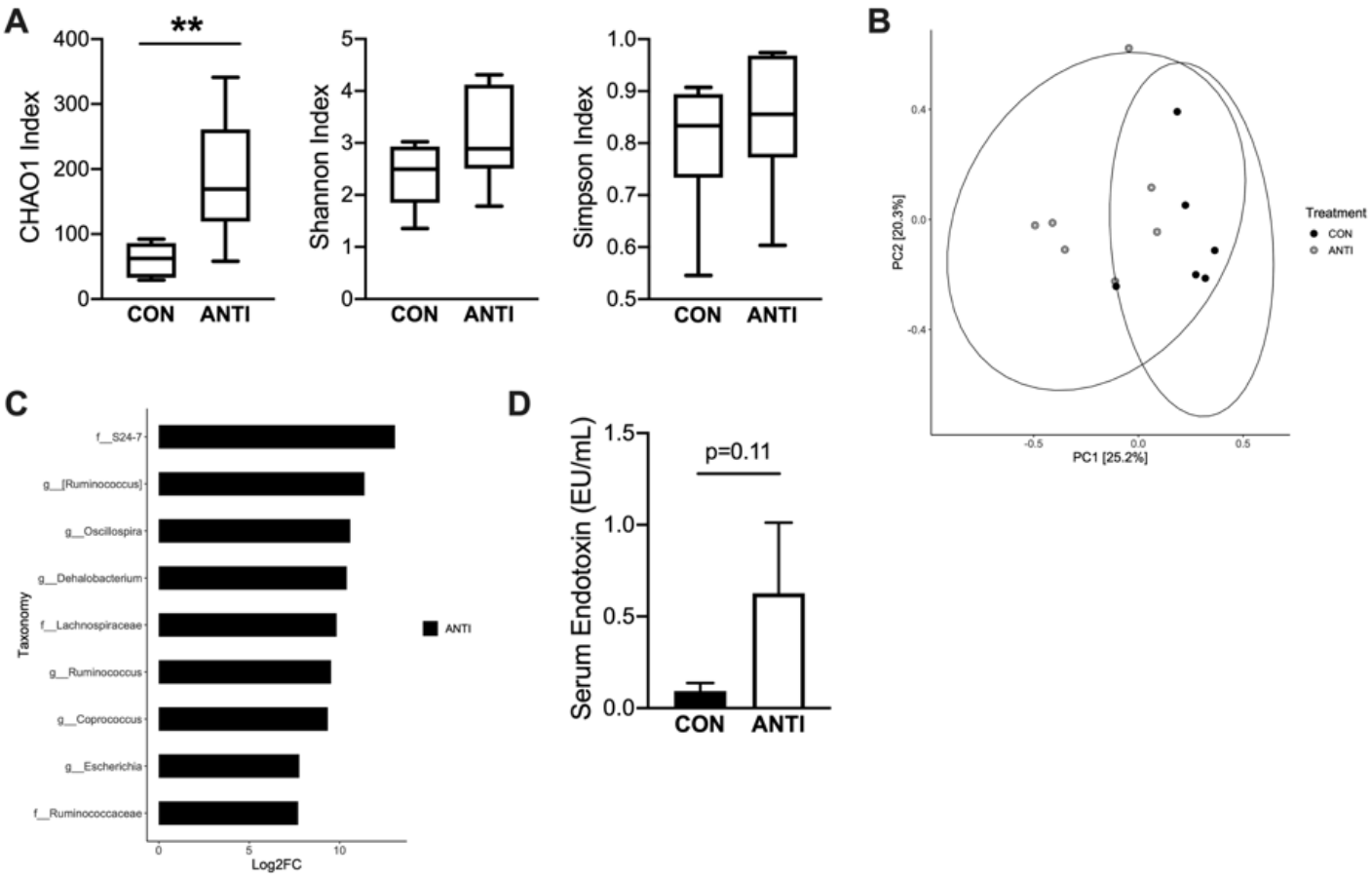


Figure 2.3.8. Gut microbial characterization in PND7 piglets. (A) Chao1, Shannon and Simpson diversity indices, **(B)** Principal component analysis showing distinct clustering of ANTI compared with CON (Adonis, p=0.026) and **(C)** Taxonomy significantly enriched in ANTI vs. CON pigs. All n=6-7 per group. **(D**) LPS concentration in blood at PND7 comparing ANTI and CON groups (n=6 per group, p=0.11) expressed by means ± SEM. ** p<0.01.

2.4. Discussion

Early life is a critical window for gut microbiota maturation and perturbations introduced by antibiotics can have long-term effects on the host's metabolism (Bäckhed, 2011; Bokulich et al., 2016; Cox & Blaser, 2015; Forgie et al., 2020). Children are susceptible to infections, therefore, studies of the early succession of intestinal bacteria and the host's development during pediatric antibiotic treatment are necessary to understand how to ameliorate potential negative outcomes (Bokulich et al., 2016; Zeissig & Blumberg, 2014). In the present study, our findings suggest that neonatal, antibiotic-induced perturbations impact key factors involved in the development and function of pancreatic β-cells, likely mediated via microbe-host interactions. The overall phenotypic differences include early changes in microbial communities that associate with up-regulation of GLP-1 signaling potential and hyper-responsiveness to glucose in β-cells along with an increase in inflammatory markers at PND7. By PND14, although islets appear to be developing normally, there is down-regulation of key regulators of development and maturation, such as *Pdx1* (which persists at PND21 (Li et al., 2017)). By PND49, the antibiotic-induced phenotype includes impaired glucose tolerance despite hyperinsulinemia (Li et al., 2017) accompanied by low islet insulin content and reduced mass of both α- and β-cells.

In the prediabetic state, β-cell compensatory mechanisms include enhanced insulin biosynthesis, secretory capacity and β-cell hyperplasia (Leahy, 2008) that help to maintain normoglycemia (Tabák et al., 2012), and failure of β-cell adaptation to metabolic demands plays a central role in the pathogenesis of type 2 diabetes. Previously, we observed that PND49 piglets exposed to neonatal amoxicillin had impaired glucose tolerance (Li et al., 2017). In the current study, ANTI PND49 piglets had euglycemia but non-fasting hyperinsulinemia despite 40% lower islet insulin content and reduced β-cell mass, which is consistent with stressed β-cells. Conversely,

and similar to results from PND21 piglets (Li et al., 2017), isolated islets from ANTI piglets at PND7 and PND14 were hyper-responsive to glucose. Furthermore, at PND21, islets from ANTI piglets had a trend to increased β-cell area and increased insulin content, suggesting that metabolic adaptations invoked by weaning are impaired thereafter. Thus, neonatal antibiotic administration appears to program β-cells to function and proliferate abnormally, which increases the predisposition to glucose intolerance as animals age.

Evidence from the intrauterine growth retardation (IUGR) model concurs that β-cell failure to adapt can be programmed early in life. Three-month-old IUGR mice fed a high-fat diet had hyperinsulinemia, and isolated islets had increased insulin content, higher response to glucose stimulation and overall increased β-cell mass but 12-month-old mice were hyperglycemic with decreased islet insulin content and reduced β-cell mass (Chakravarthy et al., 2008). The observed phenotype in our model is comparable to the early stages of β-cell dysfunction in type 2 diabetes rodent models (Chakravarthy et al., 2008; Corbin et al., 2016) and we hypothesize that in this case a gut microbial factor determined by antibiotic exposure is likely responsible.

Changes in the expression of genes that regulate β-cell development at PND21 (Li et al., 2017) led us to perform a time-series study of gene expression and islet morphology at PND7, 14 and 49. Post-natal β-cell functional maturation is characterized by β-cell mass expansion and enhanced insulin secretion (Arda et al., 2016; Helman et al., 2020; Qiu et al., 2017).

We observed that amoxicillin treatment had a temporal effect on islet transcription factors, growth factors and *GLP-1*-related genes. Islet *PDX1*, *TCF7L2* and *IGF-2* were reduced only at PND14 in ANTI pigs. PDX-1 transcriptional regulation targets multiple genes essential to the β-cell phenotype (Mosley et al., 2004). The loss of *PDX-1* is associated with diabetes development due to a loss of specific β-cell markers, β-cell function and mass (Gao et al., 2014). β-cell insulin

secretion stimulated by incretins is regulated by TCF7L2. Selective deletion of *TCF7L2* in the β-cells of mice led to an altered incretin response and decreased β-cell mass, but not α-cell mass (Mitchell et al., 2015). Also, growth factors are involved in the proliferation of pancreatic β-cells and insulin biosynthesis during early development. Serum IGF-2 was reduced in Goto-Kakizaki (GK) rat, a model of spontaneous type 2 diabetes, at 18.5 days of gestation. GK fetuses had decreased *PDX-1* expression and reduced β-cell mass compared with Wistar control rats (Movassat et al., 2007).

While we previously observed reduced β-cell apoptosis at PND21 but decreased proliferation at PND49 (Li et al., 2017) no changes in β- or α-cell proliferation and apoptosis or overall abundance were identified in ANTI pigs at PND7, suggesting that amoxicillin treatment has a delayed effect as indicated by reduced α- and β-cell area at PND49. Consistent with other authors (Nagaya et al., 2019), at PND7 we identified insulin- and glucagon-positive cells arranged in small and large clusters, and scattered individual cells distributed throughout the acinar tissue. Overall, our results suggest that neonatal amoxicillin treatment impairs the expression of key factors that regulate pancreatic β-cell morphology, function and growth that are seen at later life and possibly increase the risk of developing diabetes in adulthood. Leahy (Leahy, 2008) proposed that the progressive β-dysfunction seen in pre-diabetes and type 2 diabetes initiates during fetal and neonatal periods. Our piglet model shows that amoxicillin treatment induces islets to be hypersensitive to glucose stimulation initially, but gradually changes the β-cell morphology and function, which results in β-cell stress.

The β-cell hypersensitivity to glucose in PND7 and PND14 ANTI pigs, and increased insulin-positive cells in ANTI PND21 pigs, suggested involvement of GLP-1, an incretin that exerts beneficial effects on β-cell proliferation and function, with its analogs used therapeutically

for diabetes (Andersen et al., 2019). PND7 ANTI pigs had elevated circulating active GLP-1, concurrent with higher gene expression of *GCG*, *PSCK1*, *PSCK2*, and *DPP4*, which was not seen in older ANTI pigs or in the ileum, where GLP-1 is produced in L-cells and known to be influenced by microbial metabolites (Nguyen et al., 2014; Panaro et al., 2020; Rastelli et al., 2019)

Interestingly, germ-free mice have elevated GLP-1, although the mechanism driving this phenotype is not understood (Selwyn et al., 2015). GLP-1 receptor and enzymes involved in GLP-1 synthesis are found in α-cells from fetal and neonatal humans and pigs (Arda et al., 2016; Kim et al., 2020).

GLP-1 induces neogenesis and increases the number of single β-cells and β-cell clusters associated with pancreatic ducts in 7-old-day streptozotocin-treated rats (Tourrel et al., 2001). IUGR rats treated with the GLP-1 analog exendin-4 from PND0-6 exhibit improved glucose tolerance and remain normoglycemic whereas vehicle-treated littermates become diabetic by 8 months of age, suggesting a protective role of early exposure to GLP-1 (Stoffers et al., 2003). Thus, amoxicillin exposure triggers transient changes in *GLP-1*-related genes, which might be related to enhanced glucose responsiveness seen in antibiotic-treated piglets from PND7-21 (this study and (Li et al., 2017). Conversely, attenuation of GLP-1 at PND14 might be a factor in the apparently normalized glucose responsiveness of islets at PND49.

There is increasing evidence that alterations in the gut microbiota composition and intestinal barrier contribute to low-grade inflammation and production of pro-inflammatory cytokines (Peterson & Artis, 2014), which may elicit β-cell apoptosis and dampened insulin secretion seen in type 2 diabetes (Paniccia & Schulick, 2016). In addition, the exposure of mouse islets to inflammatory cytokines IL-6 and TNF-α induces down-regulation of *PDX1*, glucokinase (*GCK*) and *INS2* (Yang et al., 2010). Our data show that amoxicillin increased the abundance of

Enterobacteriaceae in the ileum concomitant with reduced abundance of tight junction proteins and increased circulating LPS at PND7. Translocation of LPS to the systemic circulation elicits metabolic dysregulation known as metabolic endotoxemia (Cani et al., 2012). The concurrent elevation of *TNF* and *NPG1* mRNA in ANTI islets suggests a local inflammatory and counter-inflammatory response in the pancreas. Cathelicidins such as *Npg1* are antimicrobial peptides found in the gut that selectively inhibit the production of pro-inflammatory cytokines in response to LPS (Han et al., 2016; Song et al., 2015). Cathelicidins are also more highly expressed in β-cells of diabetes-resistant than -prone rats (Pound et al., 2015) and, in NOD mice, confer local protection to β-cells from pro-inflammatory cytokine attack, an effect regulated by gut microbes via SCFA production (Sun et al., 2015). However, our study found neither an increase in SCFA in fecal material or their receptors expressed in islets suggesting an alternative means of regulation.

In conclusion, this study found that neonatal antibiotic administration leads to increased abundance of Enterobacteriaceae. We propose this leads to an early increase in gut permeability and metabolic endotoxemia, which manifests as transient but functionally significant changes in key β-cell transcription factors, associated with transient upregulation of GLP-1 synthesis and signaling, and β-cell glucose responsiveness. This is followed by a window in which genes essential in β-cell development and growth are down-regulated. Consequently, β-cell maladaptation to nutrient-induced metabolic stress later in life is observed.

Chapter 3: Early-life antibiotic exposure and *Escherichia coli* colonization in combination induce negative long-term effects on glucose metabolism

3.1. Rationale

Type 2 diabetes and prediabetes prevalence is increasing alarmingly in children and youth populations, and is ascribed to the childhood obesity epidemic (Diabetes Federation International, 2019; Sinha, et al., 2002). In Canada, the mean age of type 2 diabetes diagnosis in youth was 13.7, and 8% of all newly diagnosed children were less than 10 years of age (Wherrett & Ho, Diabetes Canada Clinical Practice Guidelines Expert Committee.2018). Diabetes appearance at early age increased the risk of developing diabetes-associated complications and cardiovascular disorders (Li et al., 2009; Wherrett & Ho, Diabetes Canada Clinical Practice Guidelines Expert Committee. 2018).

Impaired glucose tolerance and/or impaired fasting glucose have been used for early identification of children and adolescents at high risk for future diabetes and cardiometabolic disorders. Studies have identified that adolescents with impaired glucose tolerance exhibited elevated levels of proinsulin, C-peptide, and fasting plasma insulin compared to those with normal glucose tolerance or adults with diabetes (Arslanian et al., 2018; Sinha et al., 2002). Moreover, obese children and adolescents displayed severe insulin resistance and hyperinsulinemia that was not present in adults with impaired glucose tolerance (Li et al., 2009; Sinha, et al., 2002).

The increased β-cell workload to meet the insulin demand in obese, insulin-resistant youth might accelerate and enhance β-cell damage. The Early-Bird longitudinal study reported that

children who exhibited high blood glucose at 5 years of age developed impaired fasting glucose and loss of insulin sensitivity during puberty independently of the body mass index (Hosking et al., 2013). In clinical trials, the use of metformin alone or in combination with insulin glargine failed to delay β-cell dysfunction in 50% of youth participants with impaired glucose tolerance (Arslanian et al., 2018; Nadeau et al., 2018; Zeitler et al., 2012). Together, these findings suggest that in prediabetic children and adolescents, the progressive β-cell function loss is more aggressive and faster than in adults, and there is an urgency to identify the major risk factors that contribute to impaired glucose tolerance.

Along with genetic factors and excessive consumption of energy, gut microbiota can contribute to the aetiology of obesity and diabetes. Individuals with prediabetes and type 2 diabetes had low bacterial richness that is positively associated with elevated fasting blood glucose, insulin resistance, C-peptide, and HOMA-IR, which are common clinical biomarkers for diabetes diagnosis (Allin et al., 2015; Pedersen et al., 2016). Gut microbiota of individuals with type 2 diabetes has been found to differ in composition compared to healthy controls and is believed to be a contributor to impaired glucose tolerance. Gram-negative bacteria *Enterobacterioceae, Bacteroides, Erysipelotrichaceae* and *Prevotella* families from *Bacteroidetes* and *Proteobacteria* phyla are enriched in the intestinal microbiota of subjects with type 2 diabetes (Chávez-Carbajal et al., 2020; Larsen et al., 2010; Régnier et al., 2020; Wang et al., 2020). Lipopolysaccharide (LPS) is a component of the outer cell membrane of Gram-negative bacteria and have been shown to trigger inflammatory-related responses associated with insulin resistance during the onset of diabetes (Cani et al., 2007; Gaike et al., 2020; Larsen et al., 2010; Régnier et al., 2020). High-fat diet facilitates LPS translocation from the gastrointestinal tract surface to circulatory system by

inducing tight-junction proteins disruption (Boulangé et al., 2016; Régnier et al., 2020). Also, chronic fat consumption favors the increase synthesis of chylomicrons and lipid rafts, which can incorporate LPS and facilitates its translocation to the blood stream, and reach peripherical organs to initiate inflammatory responses (Rohr et al., 2020). Altogether, these mechanisms suggest that gut microbiota perturbations induced by environmental factors contribute to low-grade inflammation and insulin resistance observed during the onset of diabetes.

Antibiotics are widely used in the clinical treatment of infection caused by Gram-negative bacteria. Infants are the frequently exposed to a high number of antibiotic courses between the first 6 to 12 months (Leong et al., 2020). Epidemiological studies have reported that children exposed to antibiotics during the first year of life have an increased risk to body mass index and overweight (Ajslev et al., 2011; Mbakwa et al., 2016; Trasande et al., 2013). Notably, subtherapeutic doses of antibiotics prenatally or at weaning period in mice caused a temporary change in gut microbiota community, and upregulation of genes related to lipid metabolism in the liver that was associated with an increased adiposity later in life (Cho et al., 2013; Nobel et al., 2015). Specific bacteria taxa associated with an increased fatty acids synthesis in the liver were identified in mice exposed to antibiotic at neonatal stage and high-fat diet, suggesting that early-life microbiota has an influence in nutrient metabolism and energy storage (Cox et al., 2014).

We previously identified that early-life therapeutic amoxicillin treatment impacts the gut microbiota community, which results in changes in glucose metabolism later in life (Li et al., 2017). Neonatal piglets exposed to amoxicillin had changes in gut microbiota composition that were observed only at PND7 piglets. A significant transient increase abundance of Enterobacterioceae, largely represented by *Escherichia* were observed at PND7 piglets exposed to

antibiotics, however at later time points of life these significant changes were lost. Notably, early-life antibiotic treatment had a negative impact on the expression of key genes related to β-cell maturation and pancreatic islets growth and glucose tolerance even after antibiotic withdrawal. This findings suggest that perturbations of gut microbiota during early-life have an effect in host's metabolic program (Li et al., 2017).

LPS levels increase due to Gram-negative growth or bacteria death. Antibiotics contribute to elevated LPS in blood, caused by *E. coli* lysis, which exacerbates the immune system activity and low-grade inflammation (Sun & Shang, 2015; Van Den Berg et al., 1992). In addition to the antibiotic effect, chronic consumption of fat diet impairs gut microbiota, which impacts hepatic lipid metabolism and adiposity (Mahana et al., 2016).

We hypothesize that consumption of a diet with a high content of fat will accelerate the obese phenotype induced by early-life antibiotic administration, and hence increase the susceptibility to develop type 2 diabetes. The aim of the present study is to determine the effect of early-life antibiotic exposure in mice conventionalized with the Gram-negative bacteria *Escherichia coli* and weaned onto high-fat diet on glucose metabolism. Changes in insulin dynamics (insulin action and insulin secretion) are critical markers that predict impaired glucose metabolism and subsequent diabetes development. Identifying the mechanisms by which gut microbiota perturbations during the neonatal period increases diabetes susceptibility will contribute to the early identification of children and adolescents in risk and to propose strategies to mitigate environmental factors effect on gut microbiota shifts.

3.2 Material and Methods

3.2.1 Ethics Statement. The animal use in this study was approved by the University of Alberta Animal Care and Use Committee (AUP0000671) and performed according to the guidelines of the Canadian Council on Animal Care.

3.2.2. Animals and Experimental Design

3.2.2.1. Animal experiments. Eight-week-old germ free C57BL/6 mice (male n=8 and female n=8) housed in the University of Alberta Axenic Mouse Research Unit. Mice had free access to food and water and handled in a biosafety cabinet under specific pathogen-free conditions. All of the breeding pairs were colonized with a microbial community free of coliforms that was obtained from mice purchased from Jackson Laboratory (Bar Harbor, ME) previously used in our laboratory. The other half was exposed to a commensal *Escherichia coli* strain generated our laboratory (Ju et al., 2017). At parturition, half of the breeding pairs received amoxicillin in the drinking water. The randomly assigned pairs resulted in the 2 breeding pairs for each of the following groups: control (C), control and antibiotic (CA), *E. coli* (E), or *E. coli* and antibiotic. The offspring of these breeding pairs were the subject of the subsequent study.

3.2.2.2. Commensal *E. coli* colonization in mice and quantification in fecal samples. Mice were identified to be free of *E. coli* by selective culture of fecal samples on MacConkey agar. The commensal *E. coli* strain isolated from a healthy NIH Swiss mouse (Harlan Laboratories, Inc., Indianapolis, IN) on MacConkey agar was described previously (Ju et al., 2017). The *E. coli* was cultivated in 5 ml of Luria-Bertani (LB) medium (Fisher Scientific, Nepean, Ontario, Canada) at 37°C for 16 h. Mice were treated with approximately 2.0×10^7 CFU/ml of *E. coli* by oral gavage with 0.1 ml of culture medium. Enumeration of *E. coli* was conducted by serial dilutions of fecal

samples plated on MacConkey agar (BD, Sparks, MD), and total CFU counts per gram fecal contents were then calculated. Oral gavage with commensal bacteria (C and CA) or commensal bacteria and *E. coli* (E and EA) were performed every 7 days for two weeks.

3.2.2.3. Antibiotic treatment. The day a litter was born (postnatal day 1), treatment of the dam with amoxicillin was initiated according to its assigned group. The mothers from the antibiotic treatment groups (CA and EA) were targeted to receive 25 mg/kg/day of amoxicillin based on estimated water consumption using a concentration of 0.1667 mg/mL in *ad libitum* drinking water for 14 days. The pups were exposed to amoxicillin through the mother's milk, as it has been shown to be well absorbed and be transferred to milk (Roubaud-Baudron et al., 2019). The dosage of antibiotic selected was based on that used in previous studies with mice and estimating that a healthy mouse drinks 150 mL/kg/day of water (Cabral et al., 2019). Freshly mixed amoxicillin was provided every other day. The control groups (C, E) received water.

3.2.2.4. Exposure of weaned mice to high-fat diet. At PND21 mice were weaned onto a high-fat diet with 45% of energy from fat, 25% from protein, and 35% from carbohydrate (Research Diets Inc, New Brunswick, NJ, USA; Cat# D12451) until euthanized at PND84.

3.2.3 In vivo assays

3.2.3.1. Body weight measurement. Body weight was monitored during PND14, 28, 35, 42, 70, 77 and 84.

3.2.3.2. Glucose tolerance test. Intraperitoneal glucose tolerance test (IPGTT) was performed in 10-week-old mice after an overnight fast (16 hours). Body weight was measured, and values of fasting blood glucose were obtained using glucometer (Counter Next One, Ascencia Diabetes Care Holdings, AG, Switzerland). 75 μL of blood was collected from tail vein using

blood capillary tubes (Fisherbarand Color-coded capillary tubes, Fischer Scientific Lab, USA), centrifuged (1200 rpm, 10 min at 4 °C) and the plasma retained for fasting plasma insulin determination. Next, 2.0 g/kg/body weight of 30% glucose solution was administrated via intraperitoneal injection, and blood glucose concentration was measured at 15, 30, 60, 90 and 120 minutes.

3.2.3.3. Insulin tolerance test. Insulin tolerance tests were performed in 11-week-old mice after a fast of 4 hours. Fasting blood glucose concentration was measured with a glucometer and each mouse received 0.026 IU/kg/body weight of insulin administered via intraperitoneal injection. Blood glucose concentration was measured at 15, 30, 60, 90 and 120 minutes.

3.2.3.4. Animal termination and tissue collection. To study pancreatic β-cell development and gut microbiota composition at early life, half of the litter from all treatment groups, balanced for sex, were euthanized at PND14, while the remainder of the litters were weaned onto a high-fat diet.

PND14 and PND84 mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. Blood samples were collected by cardiac puncture in tubes containing EDTA and dipeptidyl peptidase inhibitor-4 (EMD Millipore, MA). Then, plasma was obtained by centrifugation at 3,000 x g for 10 min at 4°C and stored at -80°C. Ileal, cecal and colon contents and tissue were collected, snap frozen in liquid nitrogen, and stored at -80°C.

3.2.4 Analysis of mouse tissues

3.2.4.1. Plasma insulin measurement. Plasma insulin concentration was determined using ALPCO Mouse Insulin ELISA kit (Cat# 80-INSMS-E01, E10, version September 13,2017, Salem

USA), following manual instructions. The specificity of this kit has a <0.01 cross-reactivity with mouse C-peptide 1 and 2 according to the manufacturer instructions.

3.2.4.2.DNA extraction, library preparation and intestinal microbiota composition analysis. DNA was extracted from cecum content and fecal samples as previously described. DNA concentrations were measured using Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, USA). For amplicon library generation, the bacterial 16S rRNA gene V3 and V4 regions were amplified with gene-specific primers, using KAPA HiFi Hot Start Polymerase (Kapa Biosystems Inc, Wilmington, MA, USA), according to Illumina's 16S Metagenomics library prep guide. Operational taxonomic units (OTUs) were picked using an open reference picking strategy and Greengenes for reference. A paired-end sequencing run was performed on an Illumina MiSeq platform (Illumina Inc, San Diego, CA) using 2 x 300 cycles. Sequence data were analyzed using QIIME 2 pipeline.

3.2.4.3 16S rRNA gene sequencing analysis. Raw sequence reads processing and analysis were performed using Quantitative Insights into Microbial Ecology 2 (QIIME2 v. 2020.2.0) software (Bolyen et al., 2019). Forward and reverse sequences were trimmed at 280 and 220 bp, respectively. Filtering, dereplication, chimera detection and merging of paired-end reads were performed using DADA2 software (Callahan et al., 2016). Multiple sequence alignment and phylogenetic tree generation were performed using MAFFT multiple sequence alignment (Katoh et al., 2013) and FastTree 2 (Price et al., 2010) softwares. Taxonomy classification was performed using scikit-learn naive Bayes machine-learning classifier (Pedregosa et al., 2012; Bokulich et al., 2018) and GreenGenes 99% OTUs (v13.8) as the reference sequences database. Alpha and beta diversity analysis were performed using the diversity core-metrics function in QIIME2. Linear

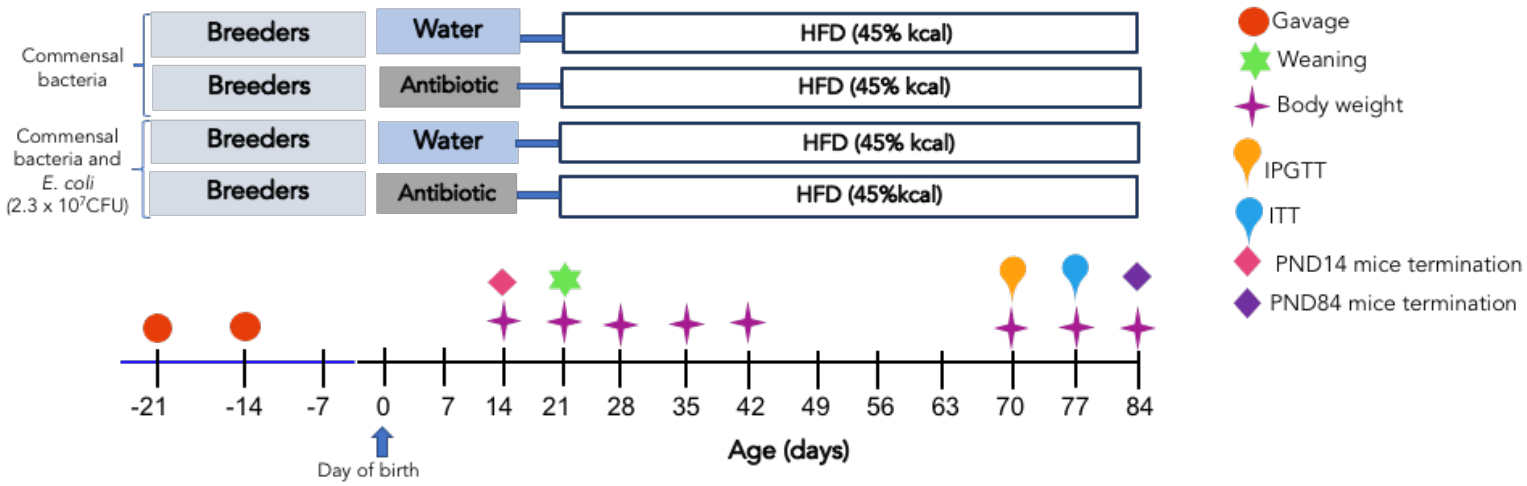
discriminant analysis effect size (LefSe) (Segata et al., 2011) was performed to determine which ASVs most likely explain differences between groups and analysis of composition of microbiomes (ANCOM) (Mandal et al., 2015) was performed to detect differentially abundant ASVs between samples.

3.2.5. Statistical analysis. Data are expressed as mean ± standard error of the mean (S.E.M). Group means were compared by Kruskal-Wallis test with a post-hoc Dunn's multiple comparison, using GraphPad Prism v6.02 software (La Jolla, CA, USA). Results were considered significantly different at $p<0.05$. P values between 0.10 and 0.05 were considered as a trend.

3.3 Results

3.3.1. *E. coli* and antibiotic treatment

Introducing amoxicillin during neonatal age increase the susceptibility to glucose intolerance (Li et al., 2017). We asked whether earlier exposure to antibiotic in combination with *E. coli* colonization exacerbate high-fat diet negative effects. In this study C57BL/6J mice colonized with commensal bacteria or commensal bacteria and *E. coli* were used as breeders. When pregnancy was detected, the dams were exposed to antibiotic or water right after the pups were born. The pups through their dam's milk received the antibiotic for 14 days, in which the last day (PND14), half of the litter was euthanized to further experiments and the other half was weaned (PND21) onto a high-fat diet for later studies in glucose homeostasis (Section 3.3.1).



3.3.1 Study design. C57BL/6 breeding pairs (male n=8 and female n=8) were colonized by gavage with commensal bacteria free of coliforms, and half were exposed to our commensal *E. coli* strain. At parturition, half of the breeding pairs were randomly assigned in the antibiotic treatment group or water group. Having in total 4 groups control (C), control and antibiotic (CA), *E. coli* (E), or *E. coli* and antibiotic. The pups were treated during the first 14 days of life. At 14-day, half of the litter was euthanized, and the other half were weaned at day 21 onto a high fat diet. At PND 77-84 glucose metabolism and insulin sensitivity assays were performed.

3.3.2. Phenotypic characterization of mice with early-life *E. coli* colonization and antibiotic exposure at neonatal and young adult stage.

We investigated whether the early-life *E. coli* colonization and single antibiotic course had an impact on metabolic phenotype of neonatal mice. (Figure 3.3.2. A). The body weight of C, CA, and EA mice at PND14 was not different, however, pups from E group displayed a body weight (7.9 g± 0.12) approximately 25% higher than pups from the C (6.1 g ± 0.20, P= 0.004) or CA (5.8 g± 0.22, p=0.01) group (Figure 3.3.2. B). During the first three weeks after weaning, E and EA mice displayed a significantly higher body weight relative to C and CA mice (Figure 3.3.2. C). However, total body weight gain was not different between groups by PND84 (Figure 3.3.2. D).

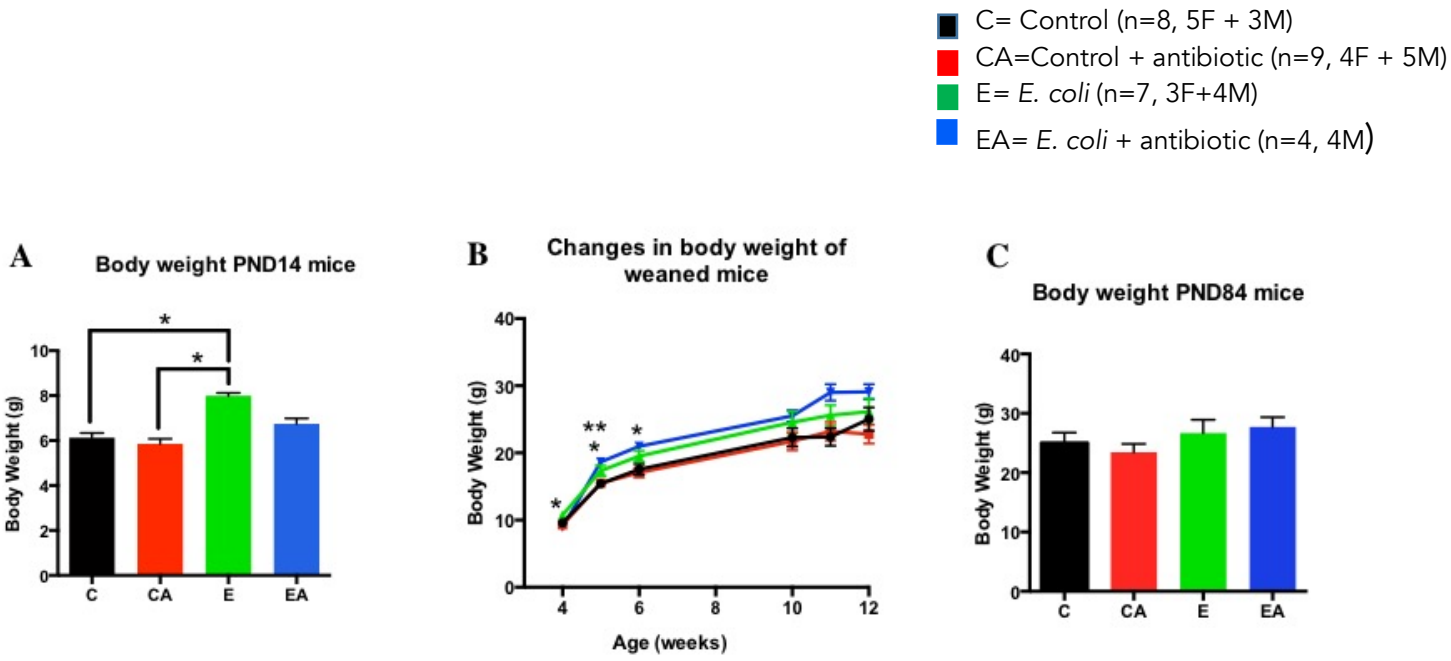


Figure 3.3.2. Body weight of mice colonized with *E. coli* and antibiotic at PND14 and PND84. **(A)** PND14 mice body weight. **(C)** Body weight over time after weaning from PND28 to PND84. **(B)** Total body weight gain by PND84 after introduction of high-fat diet at PND21. C (control; n= 8-10), CA (control + antibiotic; n=8-9), E (*E. coli;* n=6-7) and EA (*E. coli* + antibiotic; n=4) mice. Bar graphs and plots represent the mean ± S.E.M. Means were compared by Kruskal-Wallis test with post-hoc Dunn's multiple comparison test. Significant differences are represented as *p<0.05 and ** p<0.005.

3.3.3. Commensal *E. coli* in combination with antibiotic induce glucose metabolism changes.

To determine whether early-life *E. coli* colonization and antibiotic treatment increase the susceptibility to insulin resistance and impair glucose metabolism in an obesity-phenotype, we examined the dynamics of insulin secretion and sensitivity in PND84 mice. After an overnight fasting period of 16 hours, mice from EA mice (9.45 ± 0.51 mmol/L) exhibited a significant increase in blood glucose concentration compared with the mice from C (5.68 ± 0.23 mmol/L, p= 0.001), CA (6.31 ± 0.30 mmol/L, p= 0.001), and E (6.95 ± 0.41 mmol/L, p= 0.01) mice (Figure

3.3.3A). Moreover, EA mice displayed an increased fasting plasma insulin (0.52 ± 0.15 ng/mL, p= 0.03) (Figure 3.3.3. B) and HOMA-IR compared to the CA mice (32.8 ± 9.1, p= 0.03) (Figure 3.3.3. C). The HOMA-IR from C mice was lower compared to EA group, but did not reach a significant level (p=0.06).

We examined the effects of neonatal *E. coli* and antibiotic treatment on glucose metabolism. In the IPGTT, of the C mice group, blood glucose concentration reached its peak at 15 min, and thereafter began to decline through time (Figure 3.3.3. D). The blood glucose values in the CA mice followed a similar pattern as the C mice. In contrast to the C and CA groups, E mice had modestly higher blood glucose excursion in the IPGTT assay that was close to significant (p=0.058). Notably, EA exhibited an increased blood glucose excursion that was 28% and 30% higher than C (p= 0.005) and CA group (p= 0.05) (Figure 3.3.3 D) and significant differences were identified in AUC values between C (p=0.05) and CA (p=0.001) compared to EA mice, while E mice group showed a trend (0.058) compared to EA mice (Figure 3.3.3. E). In the ITT assay, treatment had a significant overall effect in insulin sensitivity (p<0.0001). However, no differences were observed between groups during the post-hoc analysis regarding single time points or in the AUC. (Figure 3.3.3. F, G). Taken together, IPGTT and ITT results suggest that the reason for a worse glucose tolerance in EA during IPGTT is not due to reduced insulin action but rather due to other mechanisms. In fact, our previous work shows that β-cell function was altered in neonatal piglets exposed to amoxicillin (Li et al., 2017) which is consistent with the present findings. All these together, suggest that in neonatal period, the colonization of *E. coli* together with antibiotic administration induce changes in glucose homeostasis in an obese phenotype, which could be in part due to impaired beta cell function.

C= Control (n=8, 5F + 3M)
CA=Control + antibiotic (n=9, 4F + 5M)
E= *E. coli* (n=7, 3F+4M)
EA= *E. coli* + antibiotic (n=4, 4M)

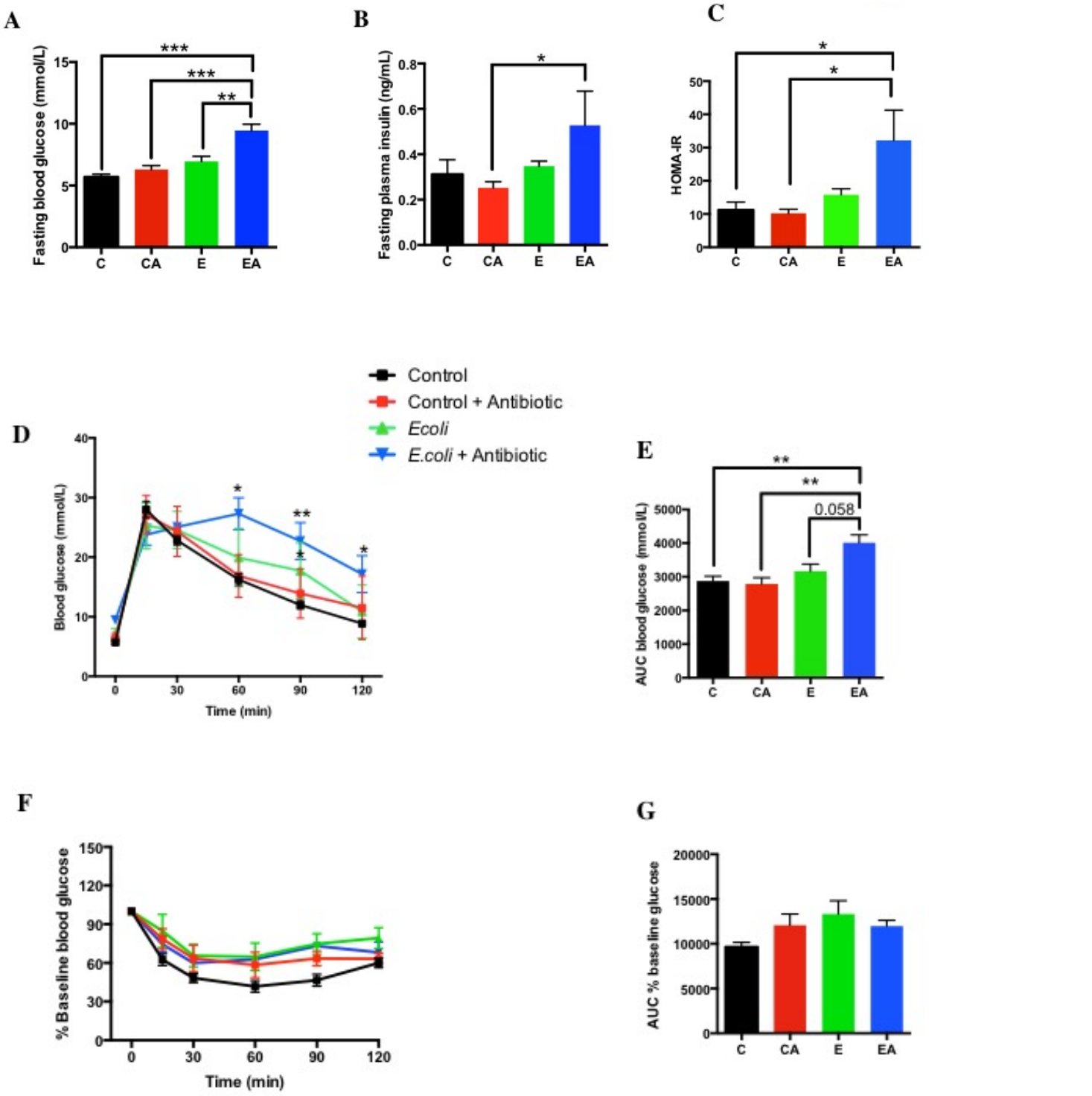


Figure 3.3.3. Impaired glucose homeostasis in mice with *E. coli* colonization treated with antibiotic at neonatal stage. PND84 mice **(A)** fasting blood glucose, **(B)** fasting plasma insulin, and **(C)** HOMA-IR values. **(D, E)** IPGTT and **(F, G)** ITT time course of blood glucose concentration and corresponding AUC analysis. Bar graphs and plots represent the mean ± S.E.M. Means were compared by Kruskal-Wallis with post-hoc Dunn's multiple comparison test. Significant differences are represented as *p<0.05, ** p<0.005 and *** p<0.001.

3.3.4. Effect of early-life antibiotic exposure on *E. coli* colonization microbiota. We observed that *E. coli* abundance was transiently depleted in the dams exposed to antibiotic (data not shown), suggesting that this effect might be observed in the pups as well. At PND 21 and 84 the AE and E *E. coli* counts did not significantly differ (Figure 3.3.4).

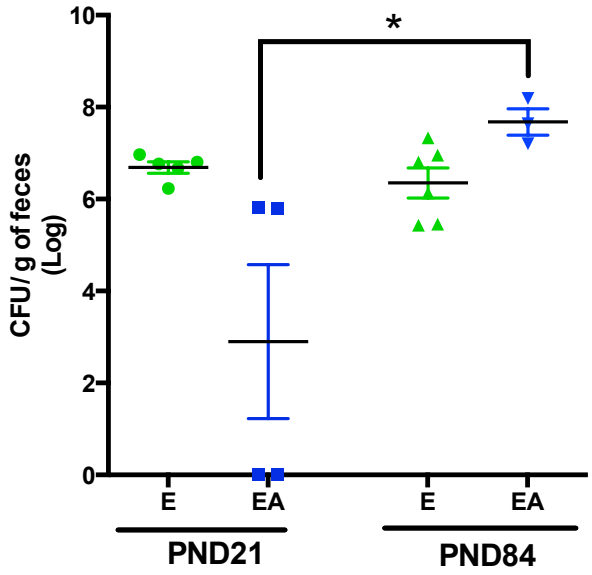


Figure 3.3.4. *E. coli* abundance is transiently depleted in mice treated with antibiotic at PND21. *E. coli* counts from E and EA mice fecal samples PND21 (first day of weaning) and at PND84. E (*E. coli;* n=6) and EA (*E. coli* + antibiotic; n=4) mice. Scatter dot plot represent individual mouse. The bar represents the mean, and the whiskers ± S.E.M. Analysis done Kruskal-Wallis, with post hoc Dunn's multiple comparisons test. Significant differences are represented as *p<0.05.

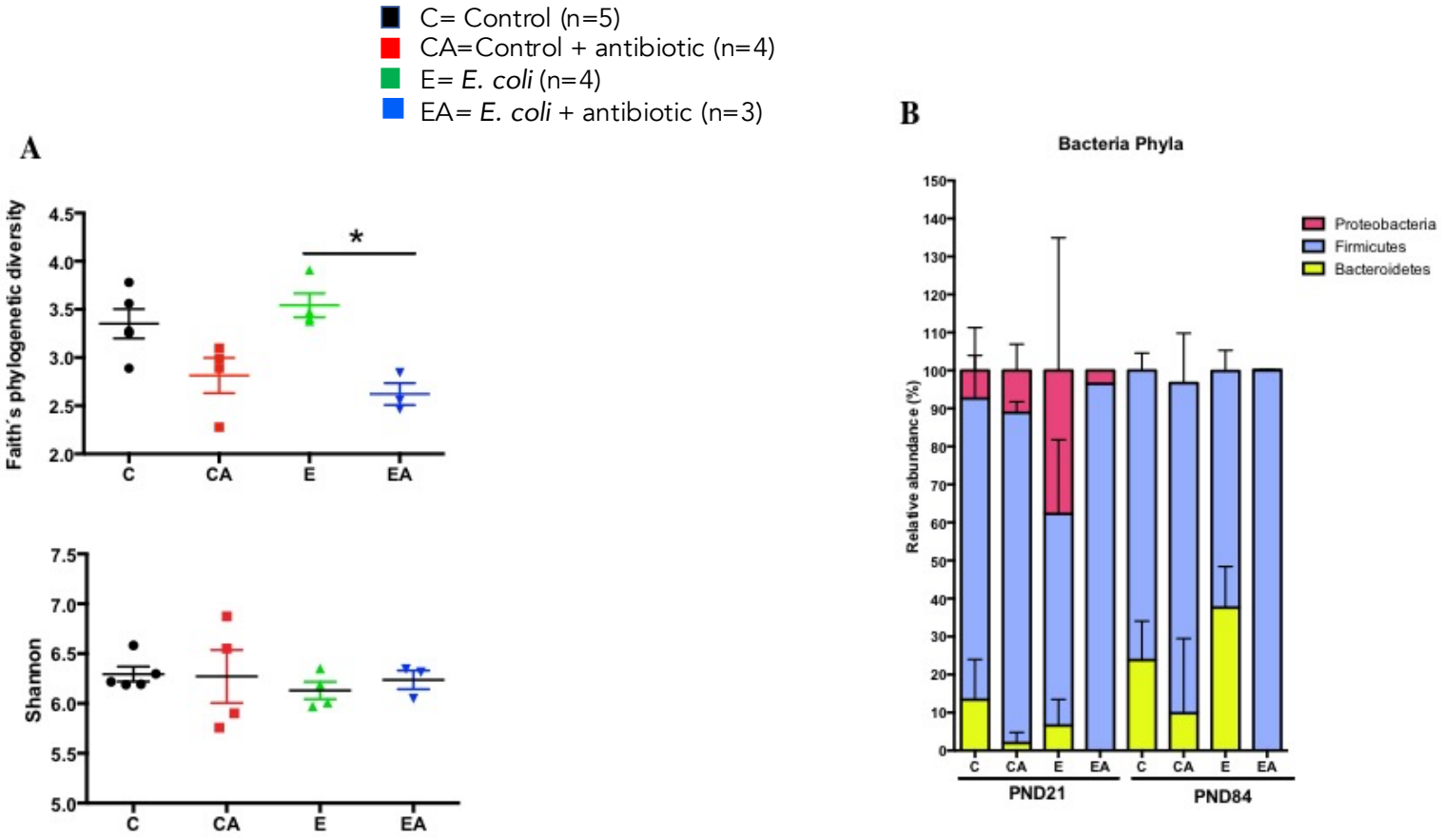
3.3.5. Effect of *E. coli* and early-life antibiotic exposure on intestinal microbiota

Breeders mice that were colonized with *E. coli* displayed a different microbial community diversity that was characterized by a low abundance of Lactobacillus, Ruminococcaceae, Lachnospiraceae, Lactobacillaceae and Bacteroidaceae (results not shown) compared to the breeders conventionalized with commensal bacteria strain.

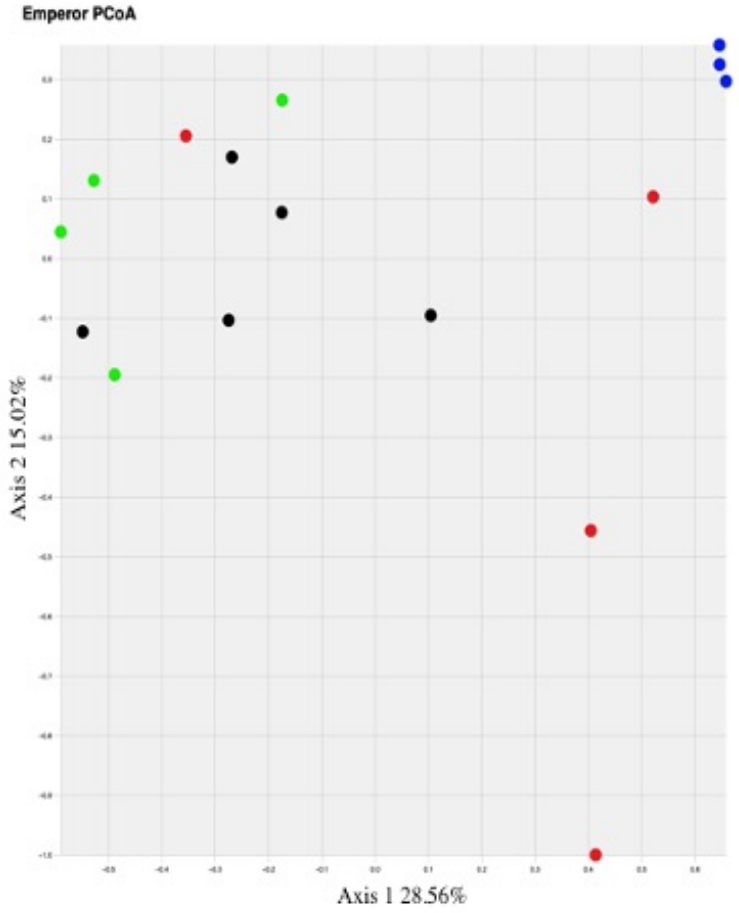
We determined whether *E. coli* and antibiotic exposure at early life could drive changes in gut microbiota community structure after mice were no longer being exposed to antibiotic. At PND84, gut microbiota community diversity expressed as Faith's phylogenetic values was significantly decreased in EA mice compared to E mice ($p=0.035$). Microbial richness and evenness measured by the Shannon Index were similar among all treatment groups (Figure 3.3.5 A). At the phyla level, C, CA, and EA PND21 mice were characterized by a high abundance of *Firmicutes* compared with E ($p=0.01$). *Bacteroidetes* was the second most abundant phylum in PND21, C mice contributing an average of 13.4%, while in E mice *Proteobacteria* was the second most abundant phylum contributing 37.7% of the microbial community. At PND84, *Firmicutes* remained as the most dominant phylum in C, CA, E and EA treatment groups. In the C, CA, and E groups, *Bacteroidetes* is the second most abundant phyla, representing averages of 23.7%, 44.1% and 37.6% (Figure 3.3.5. B). The community structure (β-diversity) in PND84 EA group was significantly distinct compared to C mice ($p=0.014$) and E mice ($p=0.034$), while dissimilarities in community structure were not identified between C, CA, and E mice (Figure 3.3.5. C). Microbial composition of PND84 CA and EA mice exhibited a reduced abundance of *S24-7* and *Peptococacceae* compared to E mice group ($p=0.031$), but not C mice. Increased abundance of

Turicibacter was observed in the EA mice that was significantly different compared to C mice (p=0.048) (Figure 3.3.5. D).

Linear discriminant analysis effect size (LefSe) and taxa abundances revealed antibiotic exposure had an impact on gut microbiota community structure that persisted in long-term in PND84 mice. *Clostridia* and *Erysipelotrichia* relative abundance increased significantly in EA group. While *Bacteroidales* and *S24-7* relative abundance was significantly increased in the E mice (Figure 3.3.5. E).



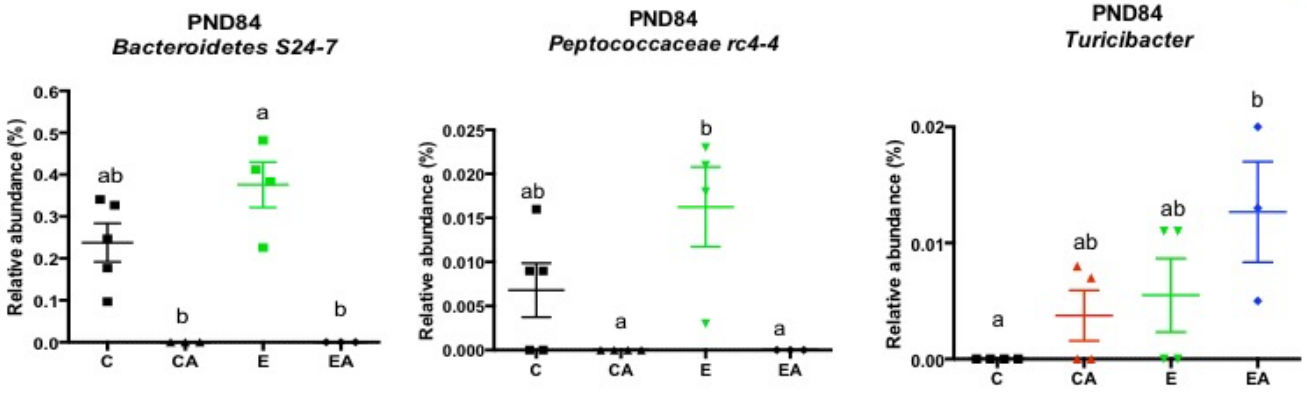
C



Group 1	Group 2	P value Pairwise Permanova
Control	Control + antibiotic	0.182
	E. coli	0.156
	E. coli + antibiotic	**0.014**
Control + antibiotic	*E. coli* + antibiotic	0.630
E. coli	Control + antibiotic	0.121
	E. coli + antibiotics	**0.034**

C= Control (n=5)
CA=Control + antibiotic (n=4)
E= *E. coli* (n=4)
EA= *E. coli* + antibiotic (n=3)

D



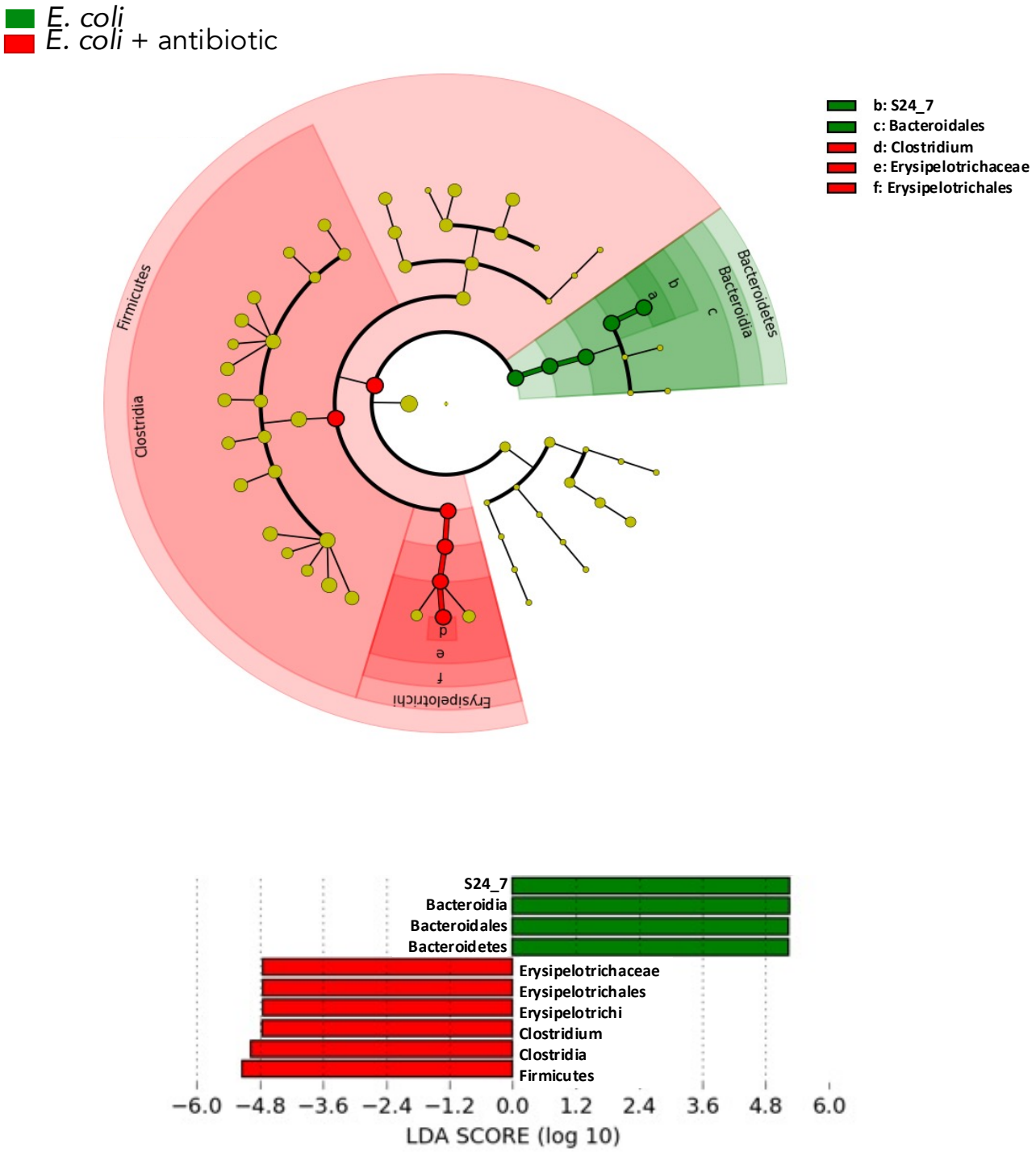

E. coli
E. coli + antibiotic
b: S24_7
c: Bacteroidales
d: Clostridium
e: Erysipelotrichaceae
f: Erysipelotrichales
Firmicutes
Clostridia
Bacteroidetes
Bacteroidia
a
b
c
d
e
f
S24_7
Bacteroidia
Bacteroidales
Bacteroidetes
Erysipelotrichaceae
Erysipelotrichales
Erysipelotrichi
Clostridium
Clostridia
Firmicutes
−6.0 −4.8 −3.6 −2.4 −1.2 0.0 1.2 2.4 3.6 4.8 6.0
LDA SCORE (log 10)

Figure 3.3.5. Characterization of gut microbiota in mice exposed to *E. coli* and antibiotic at early life. (A) PND84 mice Faith's phylogenetic diversity and Shannon values. **(B)** Relative abundance of gut bacteria at phyla level at PND14 and PND84 mice from all treatment groups. **(C)** Comparison of gut microbiota community structure between all the treatment groups at PND84. β-diversity visualized by Principal Coordinate Analysis (PCoA), using Bray-Curtis dissimilarities. Significant differences were detected using Pairwise Permanova. **(D)** Relative abundance of selected intestinal bacteria from mice at PND84. **(E)** LefSe analysis with cladograms showing significantly differential taxa between *E. coli* mice treated with water or antibiotic. Shading indicates significant overrepresentation of indicated taxa representing the specific treatment group ($p < 0.05$; LDA>2). C (control; n= 4-5), CA (control + antibiotic; n=4), E (*E. coli;* n=4) and EA (*E. coli* + antibiotic; n=3) mice. Scatter dot plot represent individual mouse. The bar represents the mean, and the whiskers ± S.E.M. For **(A)** and **(D),** the analysis was done by Kruskal-Wallis, with post hoc Dunn's multiple comparisons test. Means that do not share a letter (a, or b) are significantly different ($p<0.05$.) Significant differences are represented as $*p<0.05$

3.4. Discussion

Type 2 diabetes is a metabolic disorder diagnosed simply by the presence of hyperglycemia, yet impaired glucose homeostasis can be caused by the interaction of multiple factors (Pearson, 2019). Epidemiological and clinical studies have identified shifts in gut microbiota composition that increase the susceptibility to develop type 2 diabetes (Wu et al., 2020; Yassour, Vatanen, et al., 2016). Early-life antibiotics and dietary patterns with high-calorie content are considered as two of the major drivers of gut microbiota imbalance associated with insulin resistance (Koch, 2019; Rohr et al., 2020). However, few studies have explored the mechanism by which antibiotics at early life, in combination with post-weaning high-fat diet, induce changes in glucose metabolism that could lead to diabetes development in later life (Li et al., 2017). Hence, we aimed to investigate whether a single antibiotic course used in neonatal mice conventionalized with *E. coli* would exacerbate high-fat diet-induced glucose metabolism

impairment later in life.

Amoxicillin is a broad-spectrum antibiotic that inhibits bacteria cell wall synthesis and has limited effects on bacteria expressing β-lactamase, like members of *Enterobacterioceae* such as *E. coli*. Previous studies in infants and animal models observed a continuing abundance of *Enterobacterioceae* after broad-spectrum antibiotic exposure (Li et al., 2017; Ozkul et al., 2020). Exposure to multiple courses of antibiotic (Aversa et al., 2021; Rasmussen et al., 2018) during the first 2-4 years of life is associated with increasing prevalence of obesity (Kleber, 2010)(Sinha et al., 2002), a risk factor for developing impaired glucose tolerance and impaired fasting blood glucose, which are clinical markers used for diabetes diagnosis. We identified that body weight of PND14 E mice was significantly higher compared to C and CA mice, but not EA mice. These results suggest that the presence of *E. coli* has an impact on neonatal body weight gain.

Weaning is a critical period for growth and metabolic adjustment to diet change (Stolovich-Rain et al., 2015). Consumption of excess of energy establishes an environment of metabolic stress that accelerates β-cell dysfunction and appearance of insulin resistance (Sinha et al., 2002; Kleber, 2010). E mice at PND21 gain weight during the first 3 weeks after weaning. Notably, we observed that EA mice body weight increased in the 5th and 6th week of life and body weight gain continued over time similarly among treatment groups.

Imbalanced gut microbiota in type 2 diabetes has been associated with impaired fasting blood glucose and glucose tolerance (Wu et al., 2020). In the present study, PND84 EA mice displayed high fasting blood glucose, fasting plasma insulin and HOMA-IR values suggesting the presence of insulin resistance. Abnormal glucose metabolism was observed in the EA mice during *in vivo* studies. EA mice displayed elevated blood glucose concentration for a longer period that was

significantly different compared to C and CA mice. In addition to this, *in vivo* experiments, suggest that EA displayed a worse glucose tolerance that is not caused by a decrease insulin action, that are observed in the neonatal piglets exposed to antibiotics (Ju et al., 2017). Overall, EA displayed significant changes in glucose homeostasis compared to mice that received antibiotic alone or *E. coli* colonization, that could be explained by an impaired β-cell function. Further studies are required to examine GSIS in isolated islets from EA mice.

In the present study, changes in body weight in the EA mice suggest that *E. coli* colonization at early life might have transient effects on energy storage that is observed in body weight increase. However, a continued exposure to gut microbiota stressors (antibiotics) has a higher impact on metabolism, that results in impaired glucose homeostasis at later life, increasing the susceptibility to develop diabetes.

Antibiotic administration during the neonatal period is associated with adiposity, changes in lipid metabolism in the liver, and activation of pro-inflammatory cytokines in ileum (Cox et al., 2014). Moreover, an increase of pro-inflammatory cytokines could be induced by LPS produced by antibiotic-induced bacteria lysis and increased intestinal permeability induced by high-fat diet (Sun & Shang, 2015). Since early-life antibiotic exposure induces transient shifts on gut microbiota composition and long-term affects host's metabolism (Li et al., 2017; Zhong et al., 2019b), we asked whether *E. coli* colonization contributes to metabolic outcomes induced by antibiotic administration at early-life in an obese phenotype. In our study, we colonized breeders with commensal *E. coli* that was stable in E breeders during the experiment, however, *E. coli* counts were depleted in EA breeders and EA PND21 mice after antibiotic exposure. In contrast to our results, previous work in our lab observed an *E. coli* bloom in amoxicillin treated piglets (Li et al.,

2017), suggesting that the *E. coli* strain we used did not have amoxicillin resistance.

We found early-life antibiotic and *E. coli* colonization had no significant effects on microbial richness and evenness. We observed that early-life antibiotic exposure had a deep effect on community structure and diversity, regardless of *E. coli* colonization. In agreement with other studies, we observed that a single course of amoxicillin is sufficient to exert changes in bacterial relative abundance (Ozkul et al., 2020; Roubaud-Baudron et al., 2019). Abundance of *Bacteroidetes S24-7* and *Peptococcaceae* was reduced in mice treated with antibiotic during the first weeks of life, in both CA and EA mice. *Bacteroidetes S24-7* family stimulates colonic mucus production, a layer that protects gut barrier integrity and prevents the translocation of pathogens and microbial products (Roubaud-Baudron et al., 2019). A decrease in *Bacteroidetes S24-7* abundance induced by amoxicillin treatment at early life is associated with increased susceptibility to infection, suggesting the importance of *Bacteroidetes S24-7* as a mucus layer producer (Roubaud-Baudron et al., 2019). The reduction in *S24-7* in AE mice does not seem to be responsible for the observed impact on metabolic outcomes, as the reduction in this genus was also observed in CA mice that showed similar metabolic phenotypes as the C mice.

The present study has numerous limitations that should be addressed in the future. First, the low number of samples per treatment group, and equal distribution of females and males' limits interpretation and generalizability. The EA group was constituted by 4 males, which are prone to developing obesity in a shorter time than females. Hence, the effects observed could be confounded by sex imbalance between treatment groups, rather than a true treatment effect. Moreover, we observed that males kept alone in a cage had lower *E. coli* counts compared to those that were co-housed. Different approaches, such exchanging bedding, could help to maintain *E. coli* abundance

stability. 16S rRNA gene sequencing is a powerful tool that provides extensive information of microbiota profiles. However, the results are relative rather than absolute, so bacteria with low abundance is not included in the analysis. In our results, 16S rRNA microbiota analysis did not detect *E. coli* in any of the treatment groups (E or EA); on the contrary, we observed a high *E. coli* counts in both E and EA mice after plating on MacConkey agar. Microbial composition and metabolite analyses were unable to be completed because of the limited amount of tissue of PND14 mice.

In conclusion, we found that combined *E. coli* and antibiotic administration has long-lasting effects on glucose metabolism in mice with an obesity phenotype. We propose that the presence of *E. coli* or absence of Firmicutes members like Lachnospiraceae, Ruminococcaceae, and Lactobacillus suggest that changes in bacterial ecology in the gut can alter the metabolic interactions between different bacterial species, or between microbial species and the host, and consequently exerts effects on energy storage that favor weight gain and exacerbate the early-life antibiotic treatment.

This combination also has long-lasting effects on fasting blood glucose and fasting insulin concentration, critical values to detect insulin resistance. We observed EA mice had an impaired glucose homeostasis, suggesting that *E. coli* and antibiotic administration might affect insulin secretion, however, more studies of β-cell function need to be done.

Chapter 4. General Discussion

4.1. Summary and Conclusions

The studies in this thesis focused on examining early-life gut microbiota perturbations and their long-term effects on glucose homeostasis. By using animal models with an imbalance in gut microbiota induced by neonatal antibiotic and/or *E. coli* exposure we observed an overall change in the phenotype that included a transient alteration in gut microbiota composition that was associated with changes in the gene expression of key transcription factors and incretins involved in β-cell functional maturation and immune factors during the neonatal period, which led to an impaired glucose tolerance later in life. Although, we used different animal models (section 2.2.1 and section 3.2.2.1.), we observed effects on glucose metabolism in both pigs and mouse models in response to antibiotic treatment. Our first model involved neonatal piglets exposed to amoxicillin during the first 2 weeks of life and observed changes in the phenotype at PND7, 14 and 49. The resulting gut microbiota alteration was associated with changes in the expression of GLP-1 synthesis enzymes in isolated islets, inflammatory factors, and an altered β-cell response to glucose. At PND14 isolated islets appeared to have a normal development, however displayed a downregulation in the expression of transcription factors involved in β-cell maturation. In adult pigs (PND49) we detected low islet content and a decreased mass in both α–and β-cells and hyperinsulinemia, which were consistent with previous work in our lab. In our second model, which was early-life antibiotic and *E. coli* treatment followed to maturity in mice under long-term high-fat diet, impaired glucose homeostasis and insulin resistance was observed. Our results support the notion that neonatal antibiotic treatment compromises the stability of microbial

ecology in the gut, which impacts the host's metabolic programming. Different authors reported microbial ecology changes associated with increased hepatic steatosis, by observing an upregulation of pathways related to lipogenesis in the liver (Cho et al., 2012; Cox et al., 2014; Mahana et al., 2016; Nobel et al., 2015). While our studies identified changes in gut microbiota composition and its long-term effect on β-cell functional maturation. Hence, the studies presented in this thesis is in line with other authors showing al altered gut microbiota composition induced by early-life antibiotics, but we provide different mechanism that is relevant for diabetes pathogenesis.

4.1.1. New information about the effects of antibiotics on the interaction between the gut microbiota and host metabolism.

Several epidemiological studies reported that neonatal dysbiosis induced by antibiotic treatment increased the susceptibility to develop overweight in young children, which is a risk factor for future diabetes (Ajslev et al., 2011; Trasande et al., 2013). Studies in mice using sub-therapeutic or therapeutic doses of antibiotics at preweaning and postweaning periods have provided the causal molecular mechanisms between alterations in the gut microbial community with an increase in adiposity. In the early-life antibiotic mouse model, studies have observed an upregulation triglycerides synthesis and lipogenesis in the liver, associated with increased hepatic gene expression of *PPARγ, CD36*, and fatty acid binding protein-2 (*FABP2*) (Cho et al., 2012; Cox et al., 2014). Previous work from our lab using a swine model has provided evidence that changes in specific bacteria taxa abundance alter pancreatic islet development and function, and lead to impaired glucose metabolism (Li et al., 2017). We hypothesized that gut microbiota composition

perturbations will impact glucose homeostasis through mechanisms that involve β-cell dysfunction and proinflammatory cytokines that mediate microbe-host interaction.

Other studies have reported minimal disruption of microbiota composition induced by amoxicillin, a broad spectrum β-lactam antibiotic that is commonly prescribed to infants (Cox et al., 2014). Unlike studies in mice where antibiotic administration is followed from preweaning to adulthood (Mahana et al., 2016; Ruiz et al., 2017), a 14-day course of oral amoxicillin at a therapeutic dose during the first 2 weeks of life was used in our study to achieve gut microbiota imbalance. This more closely mimics the dose and exposure of children to treat infection (Willing et al., 2011). In the present study, we used a neonatal piglet as an animal model due to its similarity in the proportion of endocrine cell types in islets, and pancreatic islet cytoarchitecture, which are relevant for diabetes research (Renner et al., 2020). Moreover, neonatal pigs were co-housed and fed on sow's milk to mimic human infant environmental exposures.

The findings of this study revealed that a single course of antibiotics in neonatal piglets had a short-term effect on gut microbial community composition but detectable impact on host's glucose tolerance later in life. β-cell dysfunction and β-cell mass loss is one of the primary determinants of impaired glucose homeostasis which underlie diabetes (Cohrs et al., 2020). The β-cell's ability to regulate insulin secretion in response to nutrient-metabolic demands is acquired during the weaning period, and inadequate β-cell compensation to increased metabolic demands contributes to the susceptibility of future diabetes (Avrahami et al., 2020; Tixi-Verdugo et al., 2018). In the current study, antibiotic-treated pigs had β-cells that were hyper-responsive to glucose in the first weeks of life. Notably, antibiotic-treated pigs displayed hyperinsulinemia that

was associated with a decrease in β-cell mass in adulthood. The observed phenotype in neonatal piglets suggest that gut microbiota perturbations induced by stressors can negatively impact β-cell functional maturation and growth, which consequently increase the predisposition to diabetes.

Pancreatic islets from neonatal piglets exposed to antibiotic during the first week of life (PND7), exhibited an upregulation of enzymes involved in GLP-1 synthesis, however, islet cytoarchitecture and specialized endocrine cells were not affected. Notably, isolated islets of PND14 pigs had a downregulation in the expression of *PDX1, TCF7L2,* and *IGF2,* which are critical regulators of β-cell proliferation and mass expansion and have an important protective role towards metabolic stressors. Moreover, the activation of *TCF7L2* signaling pathway stimulates GLP-1 secretion, which also promotes β-cell proliferation at early stages of life. In our study, isolated islets from antibiotic treated piglets had abnormal gene expression of these critical factors that could have contributed to the aberrant changes in β-cell mass and glucose response seen in later life. Our animal model displays a phenotype that is comparable to human neonatal diabetes (Lemelman et al., 2018) and rodent models of IUGR (Ackermann & Gannon, 2007) and Goto-Kakizaki (Movassat et al., 2007) where diabetes development is associated mainly with β-cell failure that is programmed early in life, however, our model provides a novel mechanism by which altered gut microbiota composition by antibiotics is the major contributor.

Two weeks of antibiotic exposure was sufficient to alter ileal microbial community richness and structure in neonatal piglets, like the effects described in epidemiological studies in human infants after antibiotic exposure. This results was consistent with a previous study in our lab (Li et al., 2017) antibiotic-treated piglets at PND7 displayed a transient change in specific bacteria taxa abundance, which among them was Enterobacteriaceae.

Alterations in gut microbiota composition can elicit a cascade of inflammatory responses and enhance intestinal permeability. SCFA promote intestinal barrier integrity by increasing tight-junction protein gene expression, however, in early-life antibiotic model, an increased SCFA production were associated with adiposity in mice exposed to neonatal antibiotic (Cox et al., 2014). Our model does not show differences in SCFA concentration in cecal contents and SCFA receptors expression was normal in the ileum and pancreatic islets, however, we observed a downregulation of tight-junction protein expression in PND 7 pigs. The loss of tight-junction proteins facilitates the translocation of LPS, an outer cell membrane component of Gram-negative bacteria such as *Escherichia,* that triggers TNF-α gene expression via TLR-4 (Cornejo-Pareja et al., 2019). In the current study, 1-week old piglets had significantly increased LPS in blood that was accompanied with an increased expression of *TNF* and antimicrobial peptide *NPG1* genes in isolated islets, suggesting a local inflammatory response due to LPS elevation. Antimicrobial peptides have a protective function in pancreatic islets by preventing macrophage infiltration in the pancreas and proinflammatory cytokines damage to β-cells (Sun et al., 2015). Proglucagon-derived peptides, GLP-1 and GLP-2 are secreted from L-cells in the small and large intestine in response to tissue injury (Panaro et al., 2020). Several studies reported an increase of GLP-1 in the ileum in response to LPS-induced tissue injury (Lebrun et al., 2017; Nguyen et al., 2014; Panaro et al., 2020), while in contrast we reported an increase expression of GLP-1 receptor and enzymes related to GLP-1 synthesis in isolated islets, but not ileum of early-life antibiotic-treated piglets. These differences might be attributed to LPS route of administration (intraperitoneal administration, intravenously and infused by mini-osmotic pump implemented subcutaneously), adjusted doses (injection of 0.5, 1 or 2 mg/kg or infuse 300 or 1000 ug/kg) that might reach faster to distal gut and generate acute

responses to GLP-1 synthesis and secretion, while our study reflects endogenous LPS production by changes in bacteria taxa of the gut (Lebrun et al., 2017; Nguyen et al., 2014; Panaro et al., 2020).

Nguyen and authors (Nguyen et al., 2014) reported that GSIS in mice increased after LPS intraperitoneal injection or continuous infusion, however the effect was not strong in mice administered LPS with mini osmotic pumps. GLP-1 levels significantly increased after intraperitoneal injection of LPS and increased further after an oral glucose load. In our study, piglets exposed to antibiotics at early life displayed β-cells that were hyper-responsive to glucose, in parallel with an increase GLP-1 concentration in blood, that could have been induced by the transient elevation of LPS derived from Gram-negative bacteria Enterobacteriaceae. However, our study reveals the long-term effect on the host's β-cell metabolic programing, while Nguyen only explored LPS effects on GSIS.

In summary, antibiotic exposure of neonatal piglets induced changes in microbial ecology, which was characterized by an increased abundance in Enterobacteriaceae. During the first week of antibiotic treatment, transient elevation of GLP-1, immune factors and LPS in blood were detected. Later, we observed a downregulation of key factors related to β-cell maturation, that were associated with hyperinsulinemia events seen later in life.

4.1.2. New information about the effects of early-life antibiotics in combination with E. coli colonization on glucose homeostasis

Traditionally, metabolic stress induced by an excess of nutrient consumption is described as a major driver of diabetes incidence in infants (Sinha et al. 2002). Longitudinal studies identified

an accelerated and more aggressive β-cell dysfunction in children in contrast to diabetes progression in adults (Hosking et al., 2013). Hence, there is the need to identify the factors that trigger mechanisms underlying β-cell dysfunction that will enable the early detection of children at high risk to develop diabetes. Chapter 2 findings revealed that the early-life antibiotic piglet gut was enriched with Enterobacteria, which belongs to Gram-negative bacteria classification, accompanied with a transient increase in LPS production. Both LPS and Gram-negative bacteria abundance are main contributors of metabolic endotoxemia (Everard & Cani, 2013). Hence we hypothesized that early-life perturbations induced by antibiotic exposure and *E. coli* colonization will increase the incidence and severity of impaired glucose regulation induced by high-fat diet.

This study confirms and extends our knowledge of early-life gut microbiota perturbations impact on glucose metabolism later in life. Mice neonatally colonized with *E. coli* displayed the highest body weight among the treatment groups. However, when mice colonized with *E. coli* were also administered amoxicillin and then weaned onto a high-fat diet, they displayed insulin resistance as calculated by the HOMA-IR index. An altered glucose handling was seen during the IPGTT and ITT assay in the early-life antibiotic and *E. coli* mice, suggesting that other mechanisms different to insulin resistance might be induced in this phenotype. Early-life antibiotic and *E. coli* mice displayed altered glucose handling during the IPGTT assay. Even though, insulin sensitivity was affected by treatment effect in the ITT values, during post-hoc analysis the treatment effect was lost. Altogether, our data suggest, that gut microbiota is a key player in the development of impaired glucose homeostasis, one of the main clinical markers used to diagnosis diabetes in infants and adults (Arslanian et al., 2018).

The neonatal stage is a critical window for gut microbiota maturation and stability, and perturbations induced by antibiotics during this period contributes significantly to development of an obese phenotype in adulthood (Cox et al., 2014; Mahana et al., 2016). The findings of this study provide further supporting to the association of early-life antibiotic administration and the development of negative metabolic outcomes in the future. According to Cani et al (2012), animal models with a prolonged consumption of high-fat diet, is favors the development of metabolic endotoxemia and diabetes (Cani et al., 2012; Régnier et al., 2020). In the present study, early-life antibiotic exposure and *E. coli* colonization together enhanced an impaired glucose homeostasis in mature mice. Although, in this study neonatal mice were exposed to amoxicillin through the dam's milk during the first 14 days of life, we confirmed changes in metabolic phenotype after an antibiotic course during early life that are line with epidemiological studies (Aversa et al., 2021; Azad et al., 2017; Trasande et al., 2013) and rodent models (Cho et al., 2012; Mahana et al., 2016; Nobel et al., 2015).

Gut microbiota in subjects with prediabetes and diabetes is characterized by low bacteria diversity, but enrichment in specific bacteria taxa like *Escherichia* and Enterobacteriaceae (Larsen et al., 2010; Pinna et al., 2021). Nevertheless, changes in the relative abundance of Firmicutes or Bacteroidetes bacterial phyla has not been consistent in human diabetes studies (Ahmad et al., 2019; Chávez-Carbajal et al., 2020; Gaike et al., 2020), suggesting that shifts in phyla might be influenced by factors not related to the disease.

In the mouse study, amoxicillin exposure resulted in changes in microbial community structure regardless of *E. coli* presence although did not affect richness and evenness in mice with

commensal bacteria or *E. coli.* Firmicutes became the dominant phyla in preweaning mice and mature mice fed with high-fat diet, whereas Bacteroidetes were drastically depleted. In the mouse model, the microbial community did not rebound the same way as it did in the piglet model, which can be explained by the difference in housing (cohousing in piglet studies). It is likely that the piglet model better reflects the situation in children that are exposed to others that are not treated with antibiotic and future experiments in mice should include co-housing of treatment groups after the antibiotic exposure. Different studies have reported that amoxicillin treatment at pre- and postweaning has an intermediate effect on gut microbial community and metabolic-immune phenotype compared to tylosin (macrolide), another common prescribed antibiotic that elicits greater microbiota perturbations and exacerbates colitis induced by dextran sodium sulfate, hepatic steatosis later in life, and susceptibility to more severe outcomes of bacterial infection (Nobel et al., 2015; Ozkul et al., 2020; Roubaud-Baudron et al., 2019). The mild effect of amoxicillin in gut microbiota could be explained by its spectrum of activity, however stronger effects on microbial ecology are seen in pulsed-antibiotic treatment (Ozkul et al., 2020). In concordance with studies of antibiotic exposure during early life and changes in gut microbial community, we observed that under a background of a high-fat diet, early-life amoxicillin and *E. coli*, impaired glucose homeostasis is accelerated. The findings of this study sustain that gut microbiota impairment induced by environmental factors at early life could be used in combination with clinical markers, for the early detection of children at risk to develop diabetes.

4.2 Limitations and Caveats

Current findings revealed a significant role of gut microbiota stability in the optimal

maturation of β-cells during early life. Nevertheless, a few limitations apply to the work presented that restricts conclusions and introduced concepts.

Pancreatic β-cell function is highly influenced by nutrients and microbial-derived metabolites like bile acids, short-chain fatty acids, and branched aminoacids. We could not determine changes in gut microbiota-derived metabolites from neonatal piglets exposed to antibiotics, since these were not measured in blood (Chapter 2). Hence, the possibility that gut microbiota metabolites together with nutrients play a significant role in β-cell functional maturation, and early life antibiotic could have impacted the proportion of gut microbiota metabolites and contribute to impair β-cell function.

Specific experimental gaps remain, as was discussed in Chapter 3. Gut microbiota from mice can be influenced by a wide number of different factors, that include the facilities and room environment, mouse genetic background, age, and sex. Although mouse facility and animal procedures were followed as stated in the Canadian Council of Animal Care guidelines (Chapter 3, section 3.2.1) the number of successful breeders were 60% of the total group. Hence, the number of litters per treatment group were not equally distributed in all groups. Moreover, this study has a statistical limitation: different number of mice per treatment group, uneven number of females and males and litter effect could have limited the statistical power of this study, and limited treatment effect.

The *E. coli* strain used for this thesis was not resistant to amoxicillin in comparison to the neonatal piglet study (Chapter 2). Analysis from ileum samples of neonatal piglets exposed to amoxicillin was enriched with Enterobacteriaceae. Future studies in the mouse model should include both amoxicillin susceptible and resistant *E. coli* strains.

In Chapter 3, the *E. coli* bacteria count was not stable after antibiotic treatment, however it recovers gradually through time. Bacterial community profiles based on 16S rRNA sequencing can capture specific bacterial taxa shifts but has low sensitivity and limited resolution. Low abundance of *E. coli* in this study could not have been detected by 16S rRNA sequencing, making it hard to compare *E. coli* abundance before and after antibiotic use.

4.3 Future Perspectives

The work presented in this thesis provides a new perspective regarding the effect of early-life perturbations on host-microbe metabolic interaction, and its contribution to metabolic disorders development in later life. However, this thesis raises several questions for further investigation.

4.3.1 Pancreatic islet epigenetic markers induced by antibiotic exposure at neonatal stage

While we described transient changes in gene expression of enzymes involved in GLP-1 synthesis, transcription factors (Pdx1 and TCF7L2) and growth factors (Igf-1), the mechanism by which these events are regulated remains unclear. Epigenetic mechanisms like DNA methylation, histone post-translation modification and noncoding RNAs are largely involved in metabolic and immune programing (Nielsen et al., 2014). Environmental factors such as diet, maternal factors and even pathogens can trigger gene expression controlled by histone modification and leave a metabolic and immune imprinting in the host (Zhang & Cao, 2019). During the first year of life the β-cell maturation process depends on gene repression mediated by epigenetic markers. The expression of "forbidden genes" causes defects during β-cell maturation that leads to poor glucose concentration regulation. In IUGR models, epigenetics modifications in *PDX1* were identified,

suggesting that the downregulation of *PDX1* expression compromise β-cell development and favoring diabetes development later in life (Khare et al., 2016).

4.3.2. LPS/TLR-4/GLP-1 signaling pathway in neonatal pancreatic islets

The metabolic effects of GLP-1 have been extensively studied and are considered significant for lowering glucose concentration in diabetes. However, GLP-1 has an additional anti-inflammatory effect by decreasing gene expression of proinflammatory cytokines TNF-α and IL-6 from macrophages, in models that induced pancreatic islet inflammation induced by streptozotocin or high-fat diet (Lee & Jun, 2016). The activation of TLR-4 by LPS has been described in L-cells, however, an alternative mechanism of GLP-1 secretion in pancreas under the stimulation of TLR-4 has not been described.

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