UNIVERSITY OF CALGARY

Early life antibiotic and prebiotic exposure: impact on gut microbiota, metabolism and obesity risk

by

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A THESIS

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Abstract

Background Obesity is a complex disease with multiple contributing factors including the gut microbiota. Antibiotics, when administered early in life, disrupt gut microbiota development and thereby increase the risk of obesity, whereas dietary agents such as prebiotics, reduce obesity risk via several pathways including microbiota-dependent mechanisms.

Objective This dissertation examines how antibiotics when administered with/without prebiotics, alter metabolic, microbial and anthropometric outcomes in rodents and humans. Specifically, the objectives were to: 1) assess the impact of indirect (maternal) antibiotic exposure with prebiotic co-administration on obesity risk in dams and their offspring; 2) determine the impact of direct antibiotic/prebiotic exposure in young rat pups on obesity risk and other metabolic parameters; 3) analyze available human birth cohort data from the All Our Families (AOF) study and explore the association between maternal antibiotic exposure and infant BMI *z* scores.

Methods 1) Sprague-Dawley rats consumed antibiotic and/or prebiotic during their 3rd week of pregnancy and lactation and their offspring were challenged with a high-fat high-sugar (HFS) diet from 9-17 weeks of age; 2) Rat pups were administered three pulses of azithromycin with/without prebiotic diet and weaned onto a HFS diet; 3) Using the AOF dataset, the association between infant BMI *z* score and maternal antibiotic use during pregnancy or during birth was examined. In the animal studies, body composition, gut microbiota composition, and metabolic outcomes were examined in dams and their offspring. In the human cohort, the association between antibiotic use during birth (intrapartum, n=1303)/during pregnancy (n=1943) and infant BMI *z*-score was investigated.

Results 1) Maternal antibiotic use during pregnancy/lactation impairs metabolism and postpartum weight loss in dams and increases obesity risk in their offspring, which was prevented with prebiotic co-administration; 2) Direct administration of azithromycin increased body weight and impaired insulin production/sensitivity. Prebiotic co-administration normalized the impairments; 3) Intrapartum antibiotic exposure might contribute to the development of child overweight/obesity at 1 year of age.

Conclusion Our results provide evidence for the ability of prebiotic co-administration with antibiotics to prevent metabolic impairments and obesity in rats. Future clinical trials should investigate whether this is also possible in humans.

Preface

The dissertation is presented in a manuscript-based format.

Chapter 2 Sections of this chapter have been published in *Journal of Sport and Health Science.*
Teja Klancic, Raylene A Reimer. Gut microbiota and obesity: impact of antibiotics and prebiotics and potential for musculoskeletal health. J Sport Health Sci (2019) 1-9. doi.org/10.1016/j.jshs.2019.04.004.

Chapter 3 has been submitted for publication in *Gastroenterology*.
Teja Klancic, Isabelle Laforest-Lapointe, Ashley Choo, Erin N. Tuplin, Jodi E. Nettleton, Faye Chleilat, Nicole A. Cho, Alissa C. Nicolucci, Erna Alukic, Marie-Claire Arrieta, Raylene A. Reimer. Prebiotics prevent antibiotic-induced obesity risk and improve metabolic and gut microbiota profiles in rat dams and offspring.

Chapter 4 has been submitted for publication in *Gut Microbes*.
Teja Klancic, Isabelle Laforest-Lapointe, Jolene Wong, Ashley Choo, Jodi E. Nettleton, Faye Chleilat, Marie-Claire Arrieta, Raylene A. Reimer. Concurrent prebiotic intake reverses insulin resistance induced by early-life pulsed antibiotic in rats.

Chapter 5 has been submitted for publication in *Canadian Medical Association Journal*.
Teja Klancic, Amanda Black and Raylene A. Reimer. Intrapartum Antibiotic Prophylaxis Increases Body Mass Index *z* scores in the First Year of Life: Results from the All Our Families (AOF) Pregnancy Cohort

Given the manuscript-based format, there is some redundancy in the methods described and background information between chapters.

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Dedication

I would like to dedicate this thesis to my dear grandmother, Ana Uljančič, who was the most positive person I've ever met and is dearly missed.

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List of Symbols, Abbreviations and Nomenclature

ABT	Antibiotic treatment group
ABT+PRE	Antibiotic and prebiotic treatment group
Obese-ABT	Conventionalized mice inoculated with antibiotic offspring cecal matter
Obese-ABT+PRE	Conventionalized mice inoculated with antibiotic and prebiotic offspring cecal matter
Akt	Protein kinase B
ANOVA	Analysis of variance
ANS	Autonomic nervous system
AOF	All Our Families
BBB	Blood brain barrier
BMI	Body Mass Index
CI	Confidence interval
CNS	Central nervous system
CONV-R	Conventionally raised mice
CTR	Control treatment group
Obese-CTR	Conventionalized mice inoculated with control offspring cecal matter
DIO-P	Obesity-prone Sprague Dawley rats
DP	Degree of polymerization
DPP-4	Dipeptidyl peptidase-4
DXA	Dual-energy x-ray absorptiometry
FAS	Fatty acid synthase
FDA	Food and Drug Administration
FDR	False discovery rate
GBS	Group B streptococcus
GF	Germ free
GI	Gastrointestinal
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide 1
GLP-2	Glucagon-like peptide 2
GPCR	G-protein-coupled receptor
HDL	High density lipoprotein
HFD	High fat diet
HFS	High fat/sucrose diet
HOMA-IR	Homeostatic model assessment of insulin resistance
HPA axis	Hypothalamic-pituitary-adrenal axis
IAP	Intrapartum antibiotic prophylasis
IL-6	Interleukin 6
IL-10	Interleukin 10
ITT	Insulin tolerance test
IRS-1	Insulin receptor substrate 1
IRS-2	Insulin receptor substrate 2
Kcal	Kilocalories

LCS	Low-calorie sweeteners
LDP	Low dose penicillin
Lean	Lean reference dams
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
NPY	Neuropeptide Y
OFS	Oligofructose
OGTT	Oral glucose tolerance test
OTU	Operational taxonomic unit
PAT	Pulsed antibiotic treatment
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
POMC	Pro-opiomelanocortin
PRE	Prebiotic treatment group
Obese-PRE	Conventionalized mice inoculated with prebiotic offspring cecal matter
PYY	Peptide YY
qPCR	Quantitative polymerase chain reaction
SEM	Standard error of the mean
SCFA	Short chain fatty acids
STAT	Sub-therapeutic antibiotic treatment
TLR-4	Toll-like receptor 4
TNF- α	Tumor necrosis factor alpha
USV	Ultrasonic vocalization
Wks	Weeks
WHO	World Health Organization

Epigraph

“Student: Dr. Einstein, aren't these the same questions as last year's [physics] final exam?

Dr. Einstein: Yes, but this year the answers are different.”

CHAPTER ONE: INTRODUCTION

1.1 Background

Obesity is a highly complex disease that has increased in prevalence on a global scale over the past several decades. According to the Canadian Community Health Survey, 61.8% (~8.1 million) of men and 46.2% (~6.1 million) of women in Canada are overweight or obese.[1] Currently, more than 69% of adults in the United States of America (USA) are either overweight/obese[2] and more than 41 million children (under the age of 5) are overweight or obese around the world.[3] It is estimated that obesity rates in children will get worse as 60 million children are expected to be overweight or obese by 2020.[3]

Obesity is defined as a body mass index (BMI) over 30 kg/m^2 while a BMI between 25 – 29.9 kg/m^2 is considered overweight.[4] Obesity is a major risk factor for many debilitating diseases such as cardiovascular disorders (CVD), diabetes and cancer.[5] Obesity indirectly increases the risk of CVD via increased hypertension, dyslipidemia and glucose intolerance as well as directly via excess lipid deposition in myocardium leading to changes in cardiac function and structure.[6] Even a small 1-unit rise in BMI increases the risk for ischemic stroke by 4%, hemorrhagic stroke by 6%, atrial fibrillation by 4% and heart failure in men/women by 5%/7%, respectively.[2] Furthermore, obesity is a major unrecognised risk factor for cancer, overtaking tobacco as the leading preventable cause of cancer.[4] As many as 15-20% of cancer-related deaths are attributed to obesity with an estimated 84,000 cancer diagnoses yearly being linked to obesity.[5] While only 5-10% of cancers are due to genetic predisposition, 90-95% are due to lifestyle and environmental factors which includes obesity.[7] Childhood obesity is on the rise and research has shown that infant weight gain (between ages 0-1 year) predisposes children (odds ratio [OR] = 1.97; 95% confidence interval (CI) = 1.83 – 2.12) and adults (OR = 1.23; 95% CI = 1.16 – 1.30) to obesity.[8] It is therefore crucial to prevent obesity as early in life as possible in order to reduce the risk of preventable chronic diseases.

Due to the complexity of obesity, many factors contribute to its' development. Recently, the importance of microorganisms residing in our gut (gut microbiota) has been implicated in the development of obesity. Early life is a particularly sensitive time as the

gut microbiota is still developing and has not yet established a stable community.[9] Low microbial resistance (the ability of the microbial ecosystem to remain unchanged during an insult) and low microbial resilience (the ability to recover after a perturbation) define early life microbiota development.[10] After delivery, any microorganism can colonize the infant as long as it meets physicochemical requirements of the intestinal environment.[10] Candidate microorganisms could include bacteria from the skin (usually acquired during a caesarean section), bacteria from the breast milk and opportunistic pathogens which can completely change the development of the gut microbiota.[10] Since the immune system of a child is influenced by microbiota development, microbial imbalance early in life also increases the risk for immune-driven diseases such as asthma, hay fever, allergies, type 1 diabetes mellitus and inflammatory bowel disease, among others.[11] Therefore, the establishment of gut microbiota is extremely important and is influenced by many factors such as host genetics, vertical microbial transmission from mother to child at birth, mode of delivery and antibiotic exposure.[9]

Antibiotics administered early in life have the greatest impact on microbiota development.[11] Even indirect antibiotic exposure influences the health of a child via microbiota-dependent mechanisms.[11] Indirect exposures include prenatal antibiotic therapy (maternal exposure during pregnancy), maternal intrapartum antibiotic therapy (during birth) and post-partum maternal antibiotic exposure (transmitted to a child via breast milk).[11] Prenatal antibiotic exposure during the second or third trimester of pregnancy was associated with 84% higher risk of obesity, greater BMI *z* scores, waist circumference and % body fat at 7 years of age[12]. In line with this, when intrapartum antibiotics were administered during birth, a fundamental lack of early-life colonizers (i.e. *Lactobacillus*[13], *Bifidobacterium*[14]) in infants was seen. The lack of *Lactobacillus* and *Bifidobacterium* during the infant microbiota colonization process is worrisome as several studies have reported a correlation between low levels of *Bifidobacterium* spp. and obesity[15–17]. In addition, intrapartum antibiotics also target the neonatal blood stream, further interrupting microbiota seeding at birth.[14] Similar to studies evaluating indirect antibiotic exposure, three large cohort studies all reported increased risk of being overweight when children were directly exposed to antibiotics in the first 6 months of life.[18–20] Multiple exposures to broad spectrum antibiotics (macrolides) in boys during the

critical developmental window (<6 months) had the largest effect on BMI.[20] Mechanistically, direct/indirect early life antibiotic exposure contributes to obesity via reduced abundance of metabolically protective bacteria, increased microbiota-derived energy and altered hepatic metabolic signalling and/or intestinal defences.[21]

On the contrary, prebiotic intake (substrates that are selectively utilized by host microorganisms conferring a health benefit[22]) improves metabolic health by lowering body weight, fat mass, improving glucose control, reducing inflammation and increasing health promoting bacteria.[23,24] A recent single-center, double-blind, placebo-controlled study in overweight or obese children (7-12 years) demonstrated that a 16-week intervention with a prebiotic (oligofructose-enriched inulin [OI]) reduced BMI *z* score by 3.1%.[25] In addition, a 2.4% decrease in body fat with a 3.8% decrease in trunk body fat was seen in the OI group compared to the placebo group.[25] Inflammatory markers such as interleukin 6 (IL-6) decreased after OI compared to baseline, whereas in the placebo group IL-6 increased by 25% compared to the beginning of the intervention.[25] Importantly, a 19% decrease in plasma triglycerides was observed in the OI group with a significant increase in *Bifidobacterium* spp.[25] In animal studies, numerous mechanistic pathways have been described for the benefits seen in human trials. Short chain fatty acid (SCFA) production after prebiotic intake can target several tissues as transduction molecules, enhancing triglyceride hydrolysis and fatty acid oxidation and providing protection against diet-induced obesity.[26] Furthermore, increased production of satiety-hormones such as glucagon-like peptide-1 (GLP-1) in endocrine L-cells[27,28] as well as improved glucose homeostasis was seen after prebiotic administration. Enhanced gut barrier function via increased abundance of health-promoting *Bifidobacterium* spp.[28,29] and increased expression of tight junction proteins (reducing systemic lipopolysaccharide [LPS] levels[29,30]) consequently reduced chronic inflammation in animals exposed to prebiotics. Reduced serum LPS and inflammation with prebiotics have been linked to decreased hepatic steatosis, reduced cholesterol levels and improved weight maintenance[27,28]. Given the metabolic and microbial benefits associated with dietary prebiotic supplementation, it is plausible that co-administering prebiotics with antibiotic treatment could mitigate some of the obesity risk associated with early life antibiotic exposure.

To date, only one study has investigated the separate effects of early life administration of antibiotic or three distinct prebiotic treatments and this investigation was limited to describing the changes in gut microbiota in the treated suckling rat pups.[31] All treatments (prebiotic and antibiotic) changed gut microbiota composition immediately after administration with amoxicillin showing the strongest effect.[31] All three types of prebiotics tested reduced the abundance of Firmicutes, but only fructo-oligosaccharides and galacto-oligosaccharides/long-chain fructan mix increased bifidobacteria. While it is important to understand how gut microbial composition changes after separate antibiotic/prebiotic administration, it is even more important to investigate their combined effect and the long-term metabolic consequences of this early life microbial manipulation which is the focus of this dissertation.

1.2 Purpose of Research

The overall aim of this thesis was to conduct basic and clinical research examining the impact of early life antibiotic exposure on obesity risk and its' potential mitigation with prebiotic coadministration. The first animal study employed indirect exposure (maternal antibiotic/prebiotic treatment during pregnancy/lactation), whereas in the second animal study, antibiotics/prebiotics were administered directly to young rat pups. In a third study, we utilized data from a human birth cohort to investigate whether maternal antibiotic treatment during birth (intrapartum) or during pregnancy is associated with increased infant BMI *z* scores. Given the current obesity epidemic and high use of antibiotics in the pediatric population, non-invasive strategies need to be developed in order to reduce the harm when antibiotic exposure cannot be avoided. Ultimately, if manipulation of vulnerable gut microbiota with dietary strategies such as prebiotics is shown to lessen obesity risk in infants and children, this non-invasive intervention could provide an additional tool to prevent childhood obesity and ultimately improve quality of life.

1.3 Overview of separate chapters

This manuscript-based thesis contains 6 chapters. Chapter 1 provides a general introduction to the thesis. Chapter 2 is in-depth review of the literature related to

antibiotics, prebiotics and maturation of the gut microbiota. Parts of chapter 2 were published in the Journal of Sport and Health Science in a paper entitled "*Gut microbiota and obesity: Impact of antibiotics and prebiotics and potential for musculoskeletal health"*. Chapter 3 examines the potential of maternal prebiotic co-administration with antibiotics during pregnancy and lactation in rats to minimize obesity risk in the offspring. The manuscript from chapter 3 has been submitted to Gastroenterology. Chapter 4 investigates direct antibiotic exposure and the potential of prebiotic co-administration to reduce antibiotic-associated obesity risk and insulin resistance in young rats. The manuscript from chapter 4 has been submitted to Gut Microbes. Chapter 5 utilizes data from a community-based prospective low risk pregnancy cohort to investigate if maternal antibiotic exposure (during pregnancy/birth) is associated with infant BMI *z* score at 1, 2 and 3 years of age. Chapter 5 has been submitted to the Canadian Medical Association Journal. Chapter 6 provide an overall discussion of the findings from all three studies (chapter 3-5), proposes possible mechanisms for further investigation, limitations of our studies and future directions.

CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction

According to the World Health Organization, worldwide obesity has more than tripled since 1975, with more than 650 million adults living with obesity and over 41 million children under the age of 5 considered overweight or obese[32]. Obesity is associated with metabolic disorders affecting multiple organs and systems[33] and is recognized as a major risk factor for the development of type 2 diabetes, cardiovascular diseases (heart disease and stroke), musculoskeletal disorders (osteoarthritis) and certain forms of cancer (endometrial, breast, ovarian, prostate, liver, gallbladder, kidney and colon)[32,34].

Reduced to its most simplistic form, obesity is the consequence of greater energy intake than expenditure, however, intensive research over the past decades has uncovered the extremely complex etiology of obesity that encompasses a dynamic interplay between host genetic and environmental factors[34]. One of the most recent factors to be identified as playing a critical role in obesity development is the gut microbiota. Through its role in energy harvest, metabolic signalling and inflammation, the gut microbiota is now recognized as an important player in body weight regulation[35,36]. Strategies aimed at shifting the gut microbiota back to a 'healthy state' are providing new avenues of investigation for treatments aimed at helping to reduce the burden of obesity and its comorbidities.

2.2 Gut microbiota

The intestinal tract is the most densely colonised ecosystem of the human body consisting of bacteria, archaea, viruses and unicellular eukaryotes; so called the gut microbiota[37]. The number of microbes in the intestinal tract is approximately 100 trillion cells[38], estimated to be in the same order of magnitude as human cells[39]. The number of bacteria increases along the length of the gut to approximately 10^8 bacteria per gram of content in the distal ileum and 10^{11} per gram in the colon[40]. At the division level (phylum), Firmicutes (Gram-positive, anaerobic, spore-forming bacteria, mainly represented by the genera *Clostridium, Faecalibacterium, Blautia, Ruminococcus, and*

Lactobacillus[41]) and Bacteroidetes (Gram-negative, anaerobic, non-spore-forming bacteria, mainly represented by *Bacteroides* and *Prevotella*[41]) are dominant and can constitute over 90% of the bacteria present in the large and small intestine[42]. Even though other phyla such as Actinobacteria (*Bifidobacterium*), Proteobacteria (*Gammaproteobacteria* with *Enterobacteriaceae*), or Verrucomicrobia (*Akkermansia*) are low in numbers, they can have a major impact on health[43,44]. It is clear that individuals share similar core microbiota, nevertheless there are numerous differences between proportions, diversity, species, and gene functions[45]. Turnbaugh *et al.* (2009) suggested that instead of sharing a core human microbiome definable by a set of abundant microbial lineages, we might share a core gut microbiome at the level of metabolic functions[42]. The gene pool of our gut microbiota (gut microbiome) is at least 150 times larger than our own, providing us with a range of otherwise inaccessible metabolic capabilities[46]. Despite the fact that a definition of a healthy microbiota remains elusive[47], it has been established that the microbiome develops and matures over the course of infancy and childhood and reaches its adult form by 3 years of life[39]. Given the breadth of factors that influence the development of the infant's gut microbiota in the first year of life, interindividual differences in gut microbiota are significantly greater among children than among adults even though the infant's gut microbiota is dominated by fewer bacterial genera[45]. The sequence of bacterial species appearing in the first months of life is complex and many transient species emerge due to changes in the gut environment[11].This normal maturation can be disrupted leading to an imbalance in the microbial community or 'dysbiosis', which can ultimately affect obesity risk[36] and several other diseases (Figure 2.1)[48].

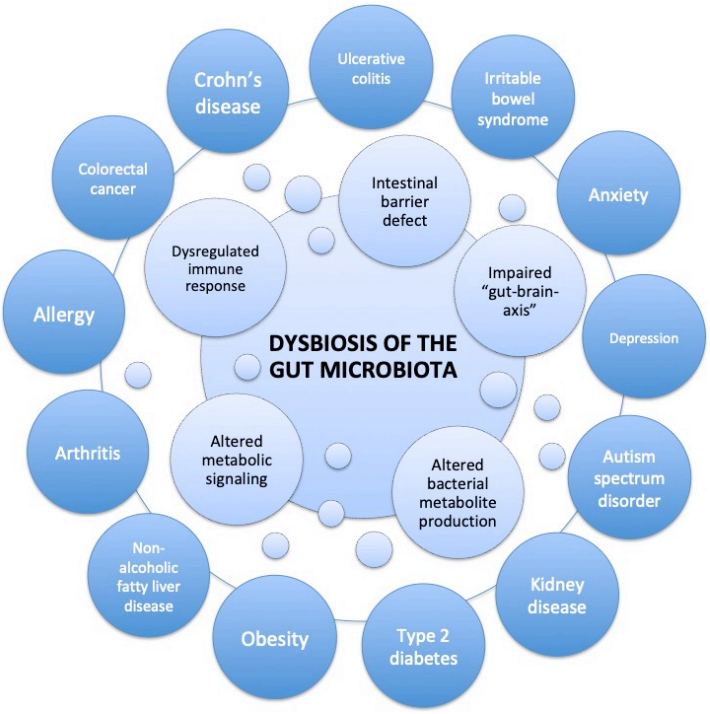


Figure 2.1 Dysbiosis of the gut microbiota in disease.

Dysbiosis of the gut microbiota impairs the intestinal barrier, immune system, metabolic functions, and bacterial metabolite production (i.e., short-chain fatty acids), as well as function/development of the central nervous system. Dysbiosis has been linked to several intestinal disorders such as inflammatory bowel disease (i.e., Crohn's disease, ulcerative colitis), irritable bowel syndrome and colorectal cancer, as well as extraintestinal disorders (i.e., obesity, type 2 diabetes, arthritis and depression).[48]

2.3 Infant gut microbiota

The microbial ecosystem has co-evolved with the host, providing the host with additional metabolic features such as vitamin K production, metabolism of non-digestible carbohydrates and xenobiotic metabolism[49]. It confers a wide-range of metabolic, nutritional, and immunological actions on the host. However, not all microbial functions are beneficial: if microbial dysbiosis occurs, microbiota can become an environmental factor contributing to obesity[50].

Several factors influence colonization of the infant gut such as gestational age (term vs. preterm), mode of delivery (vaginal delivery vs. caesarean section), infant diet (breast milk vs. formula), breast-feeding patterns[51], maternal diet, genetics, sanitation, smoking during pregnancy, familial environment (rural vs. urban), home structure (large vs. small families), geography and antibiotic treatment[52–54]. For example, 72% of the newborns' microbiota (vaginal delivery) matched species found in the stool of their mother, whereas only 41% of these species were detected in caesarean (C-section) newborns as shown by Bäckhed *et al.* (2015)[51]. Given the plethora of factors that can influence the development of the infant's gut microbiota in the first year of life, interindividual differences in gut microbiota are significantly greater among children than among adults even though the infant gut microbiota is dominated by fewer bacterial genera[45]. In addition, instability in a child's microbiota in the first 3 years of life, an increased number of infectious diseases when compared to adults and a constant exposure to new microbes (maternal skin during breastfeeding, introduction of hands and feet into their mouths) further contribute to interindividual differences between children[55]. Although it is hard to define a "normal" human gut microbiota, general trends in the maturation of infant gut microbiota can be identified from previous studies.

2.4 Prenatal exposure to microbes

Until the moment of birth, the gastrointestinal (GI) tract of a fetus is presumed to be sterile, even though some now question this hypothesis[56–60]. Aagaard *et al.* (2014) suggested that the human placenta harbours a unique microbiome resembling maternal oral microbiota, however given the culture-independent genomic approach used in this study it was not possible to determine whether the bacteria detected were viable or not[60]. In support of the sterile womb paradigm, Lauder *et al.* (2016) used a set of contamination controls (air swab/sterile swab/extraction blank), as well as oral and vaginal samples from the same women and could not distinguish between placental samples and contamination controls[61]. This finding in addition to the ability to derive axenic (germ free) animals via C-section would refute the presence of a placental microbiota. Despite uncertainties surrounding the presence or absence of live bacteria in the placenta, a recent study by Agüero *et al.* (2016) eloquently demonstrated the importance of maternal

microbial products in driving early postnatal immune development[62]. When germ-free dams were transiently inoculated with *Escherichia coli* during pregnancy alone, their germ-free offspring had an increased number of small intestinal innate lymphoid cells (ILCs) and mononuclear cells (iMNCs) compared to germ free controls[62] and maternal antibody, immunoglobulin G (IgG), was shown to be responsible for the intestinal ILC increase. Maternal antibodies were therefore shown to facilitate the penetration of microbial molecular products to the fetus driving immune system development in addition to protecting the neonate through pathogen neutralization[62]. Further support for the non-sterile environment of the placenta is provided by spontaneously released meconium from babies which harbours a complex microbial community[56–59]. In addition, bacteria could be isolated from the meconium of healthy, full term neonates using culture-dependent and culture-independent techniques[56]. Meconium released in the first 2 hours of life (prior to breastfeeding) contained enterococci (80% of the samples), staphylococci (52% of the samples) as well as *Enterobacteriaceae* (including *Escherichia coli*) and the number of different bacterial species detected in the single sample varied between 5 and 1[56]. These findings suggest that gut colonization may start before birth and prenatal exposure to fecal microbes might be a part of the "normal" development *in utero*. However, it is still unknown how these microbes gain access to the placenta. One hypothesis suggests the involvement of dendritic cells as they can penetrate the gut and directly take up pathogenic and non-pathogenic bacteria from the gut lumen[63]. Therefore, bacteria could spread to the placenta through the translocation of the mother's gut bacteria though the bloodstream[56], emphasizing the importance of understanding prenatal influences on the fetal and infant gut microbiota.

2.5 The first 2 years of life

During birth, bacteria from the mother (vaginal, fecal and skin microorganisms) and surrounding environment (air) colonize the infant gut by vertical and horizontal transmission[49]. Among the first colonizers are facultative anaerobes such as members of the *Enterobacteriaceae*[56] family until initial oxygen supplies are reduced and colonization with strict anaerobes such as *Bifidobacterium, Clostridium* and *Bacteroides* occurs[55]. During the first few weeks of life, newborn gut microbiota resembles maternal

skin and vaginal microbiota with predominant bacterial taxa including *Enterococcaceae, Streptococcaceae, Lactobacillaceae, Clostridiaceae* and *Bifidobacteriaceae*[55]. Over the next few months, *Bifidobacterium* become the dominant genus in the infant gut microbiota[64] driven by the high milk oligosaccharide content in human breast milk that selectively promotes their growth. When solid food is introduced, increases in the abundance of *Bacteroides, Clostridium, Ruminococcus* and a decrease in *Bifidobacterium* and *Enterobacteriaceae* are observed[65,66]. From 12 to 30 months of life, the composition and diversity of the infant gut microbiota becomes more stable, resembling that of an adult with *Ruminococaceae, Lachnospiraceae, Bacteroidaceae*, and *Prevotellaceae* dominating[65,67,68]. Therefore, breast-feeding and introduction of solid foods are examples of powerful environmental factors driving microbiota maturation.

Metagenomic analysis has revealed distinct patterns of genes within the microbial community at different time points in an infant's life. In newborns, genes for carbohydrate uptake are enriched followed by an increase in lactose-specific transporters at 4 months of life[51]. The microbiomes present at birth and at 4 months of life are therefore well-equipped to degrade sugars from breast milk. At 12 months of life, the microbiome becomes enriched in genes associated with degradation of complex sugars and starch, possibly due to increased intake of solid foods at this age[51]. As the infant grows older, the microbiome is exposed to an increasing array of dietary substrates that drive the microbiome to adapt to the availability of energy substrates and the environment. For example, at birth, oxygen availability in the gut enables gut microbes to use the aerobic tricarboxylic acid cycle (TCA cycle) for energy production[51] via upregulation of the gene for the enzyme responsible for converting pyruvate to acetyl-CoA in the TCA cycle[51]. Furthermore, the infant's microbiota is important for bone and heart health as enrichment of vitamin K genes is observed in newborns, which correlates with the presence of known vitamin K producers such as *Bacteroides* and *Escherichia*[51]. In addition, the newborn's microbiome soon exhibits genes for: 1) metabolism of retinol (vision, bone and teeth health); 2) folate (vitamin B9) biosynthesis (DNA synthesis and repair); 3) iron, hemin and heme (high red blood cell turnover); 4) vitamin B6 and B7 biosynthesis (nervous system development); and 5) transport of amino acids (high protein requirements). With increasing age, an increase in genes for vitamins B1, B5 and B12

biosynthesis as well as for metabolism of essential amino acids (lysine, leucine, tryptophan) are seen, reaching levels at 12 months of age that are comparable to those found in mothers[51].

It is clear that the first 3 years of life (especially the first year of life[51]) is a critical period influencing the long-term health and development of a child since this is the period when infant enteric microbiota is more viable/less stable compared to adulthood[67]. Furthermore, microbial colonization occurs in parallel with immune system maturation[69] as the epithelium serves as a barrier between the intestinal microbiota and lymphoid tissue and plays a crucial role in establishing mucosal immune response[70]. Any perturbations in initial colonization of the newborns' GI tract can lead to limited "microbial pressure" resulting in abnormal immune system maturation[71,72] causing immune mediated diseases such as allergies, multiple sclerosis, type 1 diabetes, and inflammatory bowel diseases[70,73–76]. Taken together, it is now abundantly clear that there are numerous environmental factors influencing the physiological and pathophysiological development of the gut microbiota in children and these factors are candidates for gut microbiota disruption.

2.6 Gut microbiota disruption and obesity risk

The gut microbiota of an individual with obesity may promote more efficient extraction and/or storage of energy from a certain diet, compared with gut microbiota of a lean individual. The earliest evidence supporting this hypothesis was the observation that germ-free mice are leaner when compared with conventionally raised (CONV-R) animals and transplantation of gut microbiota into adult GF mice substantially increased their fat mass despite reduced food intake[77]. In addition to more efficient energy extraction from the diet, obesogenic gut microbiota also leads to intestinal inflammation contributing to the obese phenotype[78–81]. Specifically, proinflammatory tumor necrosis factor-α (TNF-α) mRNA levels in the ileum show strong correlation with the degree of weight gain, increased fat mass and plasma glucose and insulin upon exposure to a high fat diet (HFD)[78]. Furthermore, studies showed that only CONV-R animals developed inflammation, whereas germ-free animals had no up-regulation of TNF-α mRNA levels, suggesting that HFD requires enteric bacteria to trigger intestinal inflammation.

Interestingly, only obesity-prone Sprague Dawley rats (DIO-P) and not obesity-resistant rats had increased ileal inflammation, neutrophil infiltration, and innate immune Toll like receptor 4 (TLR-4) activation once challenged with HFD[80]. In addition, DIO-P animals displayed increased intestinal permeability, favouring increased leakage of gut-derived bacterial lipopolysaccharide (LPS) into the systemic circulation, which contributes to the chronic, low-grade inflammation associated with obesity[80,81]. It is well established that LPS (a component of the outer membrane of Gram-negative bacteria)[82] and saturated fatty acids (Western diet)[83] are ligands for TLR-4 and can therefore activate the innate immune system. Upon activation of TLR-4 in several tissues (intestinal epithelial cells, adipose tissue, muscle, liver), immune cells such as pro-inflammatory M1 macrophages are activated secreting pro-inflammatory cytokines (i.e. TNF-α and IL-6).[84] Pro-inflammatory cytokines further recruit/attract more pro-inflammatory immune cells while inhibiting anti-inflammatory immune cells such as M2 macrophages and/or regulatory T cells.[84]

Chronic immune system activation and excessive production of pro-inflammatory cytokines in the tissues interfere with insulin signalling as seen when insulin and TNF-α were co-injected simultaneously into 20 lean males and insulin-stimulated glucose uptake was inhibited.[85] When mice were infused subcutaneously with LPS for 4 weeks, increased weight (whole body, liver and adipose tissue) and inflammation (i.e. TNF-α, IL-1, IL-6) were observed and the phenotypes were reproducible when mice were fed high-fat diets[86]. While an acute inflammatory response is necessary to start the healing process, there is compelling evidence that bacteria/diet-induced chronic inflammation can contribute to obesity and metabolic syndrome. Besides increased LPS production, gut permeability and chronic inflammation, bacterial dysbiosis also impacts the production of angiopoietin-like factor IV (ANGPTL4). Reduction in ANGPTL4 levels results in elevated lipoprotein lipase activity and consequently increased triglyceride deposition in the tissues such as heart, liver, adipose tissue and pancreas[77].

2.7 Gut microbiota composition in obesity

After more than a decade of research describing the link between gut microbiota and obesity, many important questions about the host-microbiota relationship remain[87].

Initially, animal studies demonstrated that obesity is associated with a change in the relative abundance of the two dominant bacterial phyla with a reduction in the abundance of Bacteroidetes and a proportional increase in Firmicutes[88,89]. Similar gut microbiota changes have been seen in adults[90] and children with obesity[91], but some studies did not support these findings[42,92] including two meta-analyses[93,94]. Accordingly, phylum-level changes in individuals with obesity are less clear mostly because of large interpersonal variation, insufficient sample sizes and the different methods employed for sequencing and quantifying taxa[94].

The most consistent finding in humans appears to be a higher abundance of *Escherichia coli* (*E. coli*) and *Lactobacillus* in individuals with obesity[95,96]. Interestingly, there are many pathogenic strains of *E. coli* (in addition to the majority of harmless *E. coli*), whereas certain strains of *Lactobacillus* are commonly used as probiotics due to their health benefits[34]. This seeming discrepancy was clarified in part by Drissi et al.[97] who reviewed evidence showing that the effects of *Lactobacillus* are age-dependent and strain-specific. With over 150 *Lactobacillus* species identified to date, this clearly represents a diverse group of bacteria[97]. Similarly, bifidobacteria are also well-known probiotics and lower abundance has been shown in individuals with higher BMI[98,99] and a negative correlation was observed between *Bifidobacterium* and visceral adiposity[98]. Likewise, lower levels of *Akkermansia* have been observed in individuals with a high BMI[100,101], however individuals with type 2 diabetes (T2D) from Asia showed increased *Akkermansia muciniphila* abundance[102]. The authors concluded that *Akkermansia* could have a beneficial impact on metabolic profiles depending on the environment in the gut. Since *Akkermansia* is a mucin-degrading bacteria it could make the intestinal barrier thinner, thereby allowing bacterial translocation and pathogenesis of T2D[102]. In line with this, a study in rodents showed that dietary fiber deficiency allows the mucin-degrading bacteria such as *Akkermansia muciniphila* to grow, express mucin-degrading enzymes and enhance disease susceptibility[103].

Regardless of inconsistencies in the precise obesogenic microbiota composition, it is clear that obesity is associated with lower diversity and richness of the gut microbiota which might compromise microbial functionality leading to disease[34]. It has been suggested that obese microbiomes can utilize a more diverse set of energy sources

resulting in greater energy harvest[104]. In order to better understand the changes in metabolism, microbial metabolites such as short-chain fatty acids (SCFA) and bile acids have to be analyzed given their role in activating signals controlling appetite[105].

Primary bile acids are synthesized from cholesterol by the liver and secreted into the small intestine where Gram-positive bacteria (mostly lactobacilli and *Clostridium* species) convert them into secondary bile acids that can act as signaling molecules[34,106]. Insulin sensitivity, energy expenditure, lipid accumulation and glucose homeostasis have all been shown to be modified by secondary bile acids which act in large part via binding to receptors such as farnesoid X receptor (FXR) and the G-protein coupled bile acid receptor (TGR5)[107]. For example, secondary bile acids can bind to ileal FXR receptors which in turn stimulate production of fibroblast growth factor 19 (FGF19) that can cross the blood brain barrier[108] and suppress activity of hypothalamic agouti-related peptide/neuropeptide Y (AgRP/NPY) neurons to improve energy homeostasis and glucose metabolism[105].

SCFAs are the end products of complex polysaccharide fermentation that can be used as an energy source by the host and can therefore influence body weight[34]. The most prominent SCFAs are butyrate, propionate and acetate; the former serving as the energy substrate for the colonocytes and the latter two being a source of glucose (gluconeogenesis) or fat (lipogenesis) in the gut and liver[109]. In obesity, higher levels of SCFAs are found in the feces of children and adults when compared to normal weight individuals[92,110] resulting from increased colonic energy harvest[111] rather than from reduced intestinal absorption[50,111]. Interestingly, SCFAs can also have a beneficial metabolic effect by improving insulin sensitivity, increasing satiety and reducing inflammation in the pancreas, muscle and adipose tissue, among others[34,109].

2.8 Gut – brain axis and body weight regulation

Behavioural changes such as altered food intake, energy expenditure and appetite modulation are also influenced by dysbiotic gut bacteria as there is bidirectional communication between peripheral tissues and the central nervous system (CNS)[112]. The bidirectional communication is enabled by neuronal, endocrine and immune linkages involving the CNS, the autonomic nervous system (ANS), the enteric nervous system

(ENS) and the hypothalamic pituitary adrenal (HPA) axis. Afferent signals arise from the gut lumen and are transmitted to the CNS, whereas efferent signals from the CNS communicate to the intestinal wall and other organs in the periphery[113]. For example, incretins are gut hormones secreted by enteroendocrine L-cells, which potentiate insulin secretion in a glucose-dependent manner. One of the most intensely studied incretins is glucagon-like peptide 1 (GLP-1) which exerts its effects by binding to G-protein-coupled receptors (GPCR). GPCRs are the largest family of membrane proteins and are communicators between the external and internal environment of cells[114]. The classical role of GPCRs is to bind hormones, neurotransmitters and other stimuli to activate specific G proteins, leading to modulation of downstream effector proteins[114]. There are 5 GPCR families and the GLP-1 receptor belongs to a small group of GPCRs (class B1)[115]. G-protein-coupled receptors are not only expressed in the pancreatic β cells, but are also present in the brain, kidney, immune cells, intestine and heart[116], therefore new actions of incretins continue to be identified.

GLP-1 is synthesized and secreted by the L cells of the small and large intestine, brain (hypothalamic, hippocampal and brainstem regions), pancreatic α cells and even taste buds[116,117]. There are two equally potent, biologically active GLP-1 forms, GLP-$1_{(7-37)}$ and the GLP-$1_{(7-36)}$ amide, with the latter representing 71% of circulating GLP-1 in human plasma[118]. Once GLP-1 is secreted into circulation, the enzyme dipeptidyl peptidase-4 (DPP-4) rapidly degrades/inactivates GLP-1, which is then cleared by the kidney. In humans, the GLP-1 receptor (GLP-1R) is a 463 amino acid heptahelical G protein-coupled receptor, widely expressed also in the peripheral and central nervous system. Therefore, in addition to its insulinotropic effects, GLP-1 exerts multiple physiological functions, including a role in reducing food intake[119]. In patients with type 2 diabetes, 6-week continuous subcutaneous infusion of GLP-1 improved glucose homeostasis by reducing appetite, fasting glucose levels and hemoglobin A_{1c}, and improving insulin sensitivity and β-cell function (measured by hyperglycemic clamp)[120]. GLP-1 analogs (i.e. Liraglutide) are an example of how basic science has been translated into clinical practice. However, their long-term safety is still ambiguous as adverse side effects are commonly reported (30-40%) and weight loss is modest (5-10%)[121]. Increasing the dose of Liraglutide increases weight loss in a dose-dependent manner, but

also promotes adverse gastrointestinal side effects[122]. A major obstacle in developing monotherapies for weight loss is that targeting anorectic pathways in the brain will result in a counter-regulatory biological adaptation of decreased energy expenditure, whereas stimulating energy expenditure will be balanced with increases in appetite[112]. For that reason, combination treatments (i.e. unimolecular triagonist: GLP-1/gastric inhibitory polypeptide [GIP]/glucagon) are under investigation in animal[123] and clinical studies[124]. Adding glucagon to incretins increases energy expenditure on top of the incretin effect.

Another way that gut bacteria indirectly/directly communicate with the brain is via LPS, an endotoxin and a major cell component of Gram-negative bacteria released from the bacterial cell wall by shedding or through bacterial lysis[125]. The highest concentration of LPS is found in the gut lumen where many trillions of commensal bacteria reside. Although even healthy individuals can have LPS present in their plasma (0 - 0.2 ng/mL)[125], a compromised intestinal barrier is permissive to much greater concentrations that can trigger downstream inflammatory cascades. LPS translocation from the gut can alter brain function via several pathways, but importantly, it can communicate with the central nervous system via cytokines that enter the brain through the blood brain barrier (BBB) or circumventricular organs and/or cytokines which sensitize vagal and spinal afferent neurons. Furthermore, systemic circulation can carry LPS to the brain directly[126], highlighting how gut microbial products such as LPS directly or indirectly modulate the activity and function of the brain.

2.9 Cesarean section and obesity risk

While many of the initial studies linking gut microbiota to obesity centered around adulthood, it is now recognized that long-term metabolic perturbations could already be initiated in early life if an obesogenic gut microbiota from mothers is transferred to the infant and/or is altered in the first years of life when microbial colonization is still in progress (e.g. from antibiotic exposure, formula feeding)[36]. In addition to antibiotics, caesarean-section also alters early microbiota development as the procedure bypasses exposure to vaginal microbiota during labor and exposes the child to skin and environmental microbes instead. For example, 72% of newborns' microbiota (vaginal delivery) matched species found in the stool of their mother, whereas only 41%

of these species were detected in C-section newborns as shown by Bäckhed *et al.* (2015)[51]. To examine the association between C-section and body mass from birth to adolescence, 10,219 children in the United Kingdom (of which 9.06% were delivered by a C-section) were investigated[127]. By 6 weeks, children born by C-section had greater weight-for-length *z* score, a phenotype which persisted until 15 years of age[127]. Similarly, in 7 year old, 46% higher obesity risk was observed in children born by C-section when compared to vaginally delivered children[12]. Unlike in human C-section studies where perinatal antibiotics are used during a C-section and confound the independent effects of birth mode, Martinez *et al.* (2017) performed a study in mice to investigate the impact of antibiotic-free C-section on early life microbiota and obesity risk[128]. Mice born via C-section gained 33% more weight at 15 weeks of age and female mice showed an even stronger phenotype (70% higher weight gain); a finding also reported previously in humans[129]. In addition to increased fat and body mass, microbiota development was altered in C-section mice[128]. Under-represented taxa in C-section animals included *Bacteroides*, *Ruminococcaceae*, *Lachnospiraceae,* and Clostridiales (associated with lean phenotypes in mice[130]) and overrepresented taxa included S24.7, *Lactobacillus*, and *Erysipelotrichaceae*[128].

2.10 Antibiotic exposure early in life

Antibiotics are the most prescribed therapeutic agents in infants worldwide[131]; they are life-saving drugs that have considerably reduced the rates of human death and disease and were thought to have minimal long-term adverse metabolic effects. However, recent research suggests that the changes that occur in our gut microbiota with exposure to antibiotics can increase the risk of obesity later in life[21]. Two types of antibiotics are particularly relevant and are described below.

2.10.1 Penicillins

Penicillins are the first choice antibiotics for most common childhood infections worldwide[131]. This class of antibiotics includes penicillin and penicillin-based antibiotics such as amoxicillin, ampicillin, cephalosporin, monobactams, carbapenems and β-lactamase inhibitors[132]. Penicillin G (benzyl penicillin) is a naturally derived beta (β)-

lactam antibiotic inhibiting the final stage of bacterial cell wall synthesis by attaching to penicillin binding proteins (PBPs) and activating cell lysis[133]. Modifying PBPs is a known mechanism for penicillin resistance used by bacteria such as pneumococci, MRSA and enterococci[134,135]. It has a broad spectrum of activity (targeting Gram-positive bacteria and some Gram negatives[133]) and a low toxicity and is therefore widely used to treat skin, ear, sinus and upper respiratory tract infections[132]. Penicillin G is commonly employed during pregnancy and lactation, because even though it crosses the placenta entering the fetal compartment (therapeutic levels can be detected in amniotic fluid) and is excreted in human breast milk, it has no teratogenic potential[133].

2.10.2 Macrolides

Macrolides (i.e. azithromycin) are the second most common class of antibiotics used immediately after penicillins[136]. The mechanism of action of azithromycin is inhibition of protein synthesis by binding to 50S ribosomal subunit, thus preventing bacterial growth[136]. Azithromycin contains a methyl-substituted nitrogen in the lactone ring and was synthesised in the 1980s as a derivative of erythromycin[136]. This change in chemical structure resulted in improved acid stability and oral bioavailability of azithromycin when compared to erythromycin[137]. The basicity of azithromycin led to quicker entrance into bacteria resulting in greater toxicity against Gram-positive bacteria[138]. With a broad spectrum of activity, long half-life (70 hours) and high cell penetrating ability (particularly phagocytes), azithromycin is very efficient for upper and lower respiratory tract infections[139]. The short duration of treatment and once per day dosing makes azithromycin well tolerated in children and adults.

2.11 Animal models and antibiotic exposure

Early experiments in pigs showed that low dose (subtherapeutic) administration of antibiotic (orally and/or injected) promoted growth of farm animals, however, the mechanisms by which it occurred were unknown[140]. Subsequent experiments in animal models have uncovered a mechanistic link between increases in body weight and subtherapeutic-dose antibiotic exposure in farm animals. Administering several types of antibiotics to mice[141] early life was confirmed as a particularly critical period for

programing of host metabolism[130] with high and low doses of antibiotics administered leading to weight loss[142–144] and gain[130,141], respectively.

Administering high-doses of antibiotics led to extensive reductions in microbiota populations that altered immunological signaling and/or decreased microbiota derived calories, thereby initiating underdevelopment and stunted growth[142]. On the other hand, exposing mice to low doses of penicillin (LDP) altered their gut microbiota with increased production of short-chain fatty acids indicating enhanced metabolism in these animals and resultant weight gain[141]. Cox *et al.*[130] demonstrated that LDP delivered via drinking water enhanced the effect of high fat diet-induced obesity and even though the microbial communities recovered after termination of LDP, the metabolic phenotype persisted. The effect was the strongest in male pups (increases in fat mass), when dams (shortly before birth and through weaning) received LDP so the pups were initially colonized with an altered maternal microbiota and further exposed to LDP while nursing.

Post-weaning exposure to LDP showed similar trends, but had a lesser effect on body composition especially in female pups where no differences were seen in fat mass when compared to control animals[130]. Interestingly, increased bone mineral content was associated with LDP exposure in female pups, whereas the opposite (decreased mineral content) was seen in males. This finding demonstrated the vulnerability to microbiota disruption in infancy and sex specific differences in phenotypes. Furthermore, they showed that LDP and high fat diet had independent effects on gut microbiota composition with *Rikenellaceae* and *Lactobacillus* (*L. reuteri* and *L. vaginalis*) being markedly reduced with LDP. Importantly, LDP exposure led to an obesogenic microbiota (increases in Firmicutes:Bacteroidetes ratio) followed by increased visceral fat accumulation and hepatic lipogenesis, reduced non-fasting serum peptide YY and a trend towards increased leptin; alterations typically seen in obesity[130]. In addition, once LDP-treated microbiota was transferred to germ-free mice, the growth promotion phenotype was transferred with it demonstrating the crucial role of altered microbiota in growth promotion. Similarly, in humans, when a 32-year-old female received a fecal microbiota transplant (FMT) from a healthy, but overweight donor, she developed obesity after FMT with her BMI increasing from 26 to 33 kg/m^2 despite exercising and dieting.[145] In another study, microarray gene expression analysis revealed that early life exposure to broad-

spectrum amoxicillin-based antibiotic delayed the maturation process of the intestine in 10-30% of genes, downregulated the genes involved in the immune system (antimicrobial products; antigen presentation) and consequently interfered with gut barrier function[146]. The authors suggested that the observed alterations in the immune system might predispose animals to increased risk of contracting infections later in life, but further studies are necessary to confirm this.[146]

One such recent study employed a mouse model mimicking pediatric antibiotic use.[147] They employed early life pulsed antibiotic treatment (PAT) using amoxicillin, tylosin or a combination of both. Interestingly, tylosin (macrolide) had worse gut microbiota profiles with delayed microbiota maturation and increased fat mass when compared to amoxicillin, whereas amoxicillin treatment displayed minimal disruption.[147] Specifically, after a week of switching animals to a HFD, amoxicillin did not differ from controls, however the tylosin group remained significantly different from controls with respect to *Erysipelotrichaceae, Ruminococcaceae, Rikenellaceae, Bacteroidales* and *Bacteroidetes*. Furthermore, only tylosin PAT increased micro- and overall hepatic steatosis later in life showing the importance of the class of antibiotics administered.[147] Taken together, animal studies suggest that direct/indirect exposure to antibiotics early in life: a) changes the gut microbiota composition; b) interferes with the development of the immune system, c) leads to change in metabolic profiles of the offspring and d) might increase the risk of obesity later in life[130,146].

2.12 Epidemiological studies in humans and antibiotic exposure

Similar to that seen in animal models, exposure to antibiotics during pregnancy and/or in the first year of life in humans is especially relevant because a stable bacterial community has not yet developed in an infant and the susceptibility to long-term problems is higher. An exclusively breast-fed infants' microbiota is dominated by bifidobacteria and research has shown that these bacteria are typically susceptible to the majority of clinically relevant antibiotics such as penicillin[148]. Therapy with broad-spectrum antibiotics is frequently observed in pediatric practices; on average, a child in the USA has received nearly three courses of antibiotics by the age of two years, about 10 courses by the age of 10 years and around 17 courses by 20 years of age[21]. Furthermore,

more than 50% of pregnant women in the USA receive antibiotic treatment during pregnancy, mostly to prevent neonatal sepsis by group B streptococcus (GSB)[39]. There are several risk factors for GSB such as smoking, previous abortion, recent sexual intercourse and current yeast infection[149]. The use of newer broad-spectrum antibiotics (e.g. amoxicillin, ampicillin) has been steadily increasing in the UK[150] as well as other developed countries and the number of infants receiving antibiotic treatment in the first year of life in Europe ranges from 18% in Switzerland to 55% in Italy, even though the frequency of illnesses does not differ between the countries[151]. Prescription rates for antibiotics vary markedly, not only within countries, but also around the world with Sweden having a 53% lower rate of antibiotic prescription than the U.S.A.[152] Interestingly, a recent survey has shown that nearly 50% of antibiotic prescriptions for children given by physicians are unnecessary[153] with amoxicillin being the most frequently prescribed antibiotic in Canada and the Netherlands[131].

In a study of Canadian infants exposed to antibiotics in the first year of life, an increased likelihood of obesity and elevated central adiposity at 9 years and 12 years of age was seen[154] and three other large cohort studies involving 28,354 mother-child pairs[18]; 11,532 children[19]; 6114 boys and 5948 girls[20] all reported increased risk of being overweight when children were exposed to antibiotics in the first 6 months of life.[18–20] Analysis of 28,354 mother-child pairs[18] from the Danish National Birth Cohort found an increased risk of childhood overweight in antibiotic-exposed infants born to normal weight mothers (OR: 1.54, 95% CI: 1.09 – 2.17).[18] A total of 11,532 children from the UK (Avon Longitudinal Study of Parents and Children) demonstrated that only the earliest window (<6 months) of antibiotic exposure was consistently associated with higher BMI *z* scores in children (+0.067 and +0.049 BMI *z* scores unit increase at 38 months and 7 years, respectively).[19] Later exposures in infancy (6-14 months and 15-23 months) were not associated with increased childhood BMI *z* scores highlighting the importance of antibiotic exposure during the earliest, critical developmental window.[19] In line with this, Saari et al. (2015)[20] found that antibiotic exposure in the first 6 months/or repeated exposures had the strongest impact on BMI *z* scores, particularly in boys (OR: 1.34, 95% CI: 1.06 – 1.66), with multiple exposures further increasing the risk of obesity.[20] When they subcategorized antibiotics by type, broad spectrum macrolides

(boys exposed <6 months; OR: 1.65, 95% CI: 1.09 – 2.31) had the largest effect on BMI.[20] As Saari *et al.* (2015)[20] reported, a longitudinal cohort study in the USA involving 64,580 children[155] concluded that multiple exposures to broad-spectrum antibiotics (in the first 24 months of life) were associated with higher risk for obesity later in life. A retrospective cohort study of 21,714 children from the UK found an association between children exposed to antibiotics in the first 2 years of life and increased risk of obesity at 4 years (odds ratio [OR] = 1.21, 95% CI: 1.07 – 1.38) with odds ratios increasing with repeated antibiotic exposures.[156] In a meta-analysis of 10 randomized controlled trials in prepubertal children (1 month to 12 years) in low and middle income countries, the conclusions were similar.[157] Their random effects model estimated an average effect for weight of 23.8 g/month (95% CI: 4.3 – 43.3) in the antibiotic treated group and the effect was stronger in children under 2 years of age.

As with postnatal exposure, prenatal (during pregnancy) antibiotic exposure also increases the risk of overweight in children. A study of 436 mother-child pairs reported an average of 84% increased risk of obesity in children at 7 years of age when their mothers received antibiotics in the second or third trimester of pregnancy[12]. Likewise, a population-based study in Denmark has shown that women who received amoxicillin during pregnancy tended to give birth to infants with higher birth weights[158]. Despite strong evidence from several studies, not all human studies agree with an association between early life antibiotic exposure and obesity[159–161]. Nevertheless, maternal intrapartum antibiotic exposure, which is largely given to prevent maternal infection during C-section birth and inhibit vertical transmission of group B streptococcus (GBS) during labor and delivery, led to impaired microbiota maturation in infants seen by a bloom in proinflammatory *Enterobacteriaceae* in the first year of life.[162,163] Since an increase in *Enterobacteriaceae* has previously been linked to increased adiposity in toddlers, it will be important in future studies to investigate whether intrapartum antibiotic exposure is associated with increased BMI *z* scores in infants and children. Birth cohorts, such as the All Our Families (AOF) low risk pregnancy cohort established in Calgary, Alberta will be valuable in answering questions related to antibiotic exposures and outcomes in children that remain unanswered.

The normal maturation of the infant and child gut microbiota is marked by important taxonomical milestones that can be perturbed by environmental factors. Bokulich *et al.* (2016) investigated the establishment of microbial communities in children in the first 2 years of life in the context of early disturbances such as antibiotic exposure[164]. A predictable pattern in the composition was seen with *Enterobacteriaceae* dominating in the first month of life followed by *Bifidobacterium, Bacteroides, and Clostridium*. With the introduction of solid foods at 6 months of life, gradual succession of several taxa lead to Clostridiales (*Lachnospiraceae, Faecalibacterium*) prevailing in the infant microbiota[164]. Antibiotics disrupted this process by changing the trajectory of α-diversity – reducing Clostridiales and *Ruminococcus* from 3-9 months of life. Using a model consisting of 22 key operational taxonomic units (OTUs), children exposed to antibiotics showed delayed microbiota maturation when compared to a reference group (vaginally delivered, breast fed, no exposure to antibiotics)[164]. Delayed microbiota maturation in the antibiotic group was due to depletion of certain OTUs such as *Lachnospiraceae*; the effect only observed during months 6-12. These findings show that during the development of a child's microbiota, antibiotics interfere with the initial low diversity and instability of the microbiota (1-12 months of life) and can delay microbiota maturation. Reducing the numbers of butyrate-producing *Lachnospiraceae* might compromise gut permeability as butyrate is the energy source for the intestinal epithelial cells and/or impair host immune system development by insufficient induction of T regulatory cells[164]. As the authors did not survey relevant health outcomes[164], it is unknown whether these transient changes in microbiota early in life impose a threat on host health in humans, nevertheless research using animal models[130,141] and human association studies[12,18–20,154,155,158] would suggest so.

The weight gain observed after early life antibiotic treatment is more pronounced in boys[18,20,154,155,165] and has been linked to a reduced abundance of metabolically protective bacteria, increased availability of microbiota-derived energy and altered hepatic metabolic signaling and/or intestinal defenses[21]. In addition to obesity, low bacterial diversity early in life increased the risk of developing asthma[166] and type 1 diabetes later in life[167]. Children developing type 1 diabetes had a 25% reduction in α-diversity and their microbiota actively promoted a metabolic environment favoring

inflammation and pathogenesis prior to disease onset (prediabetic stage)[167]. Increased relative abundance of *Blautia*, *Ruminococcus* and *Streptococcus* genera, which can act as pathogens and reduced abundance of *Lachnospiraceae* and *Veiloonellaceae*, was seen. A positive correlation was detected between *Blautia* and *Ruminococcus* with plasma triglycerides and branched-chain amino acids; both commonly elevated in patients and mouse model of diabetes[168]. Metagenomics analysis revealed a shift in microbial function from the synthesis of nutrients to the passive transporting of nutrients, favoring the growth of auxotrophic organisms, which thrive in inflammatory environments[167]. So even though type 1 diabetes is an autoimmune disorder resulting from the T-cell mediated β-cell destruction, gut microbiota can contribute to the development of the disease[169]. In line with this is evidence from a randomized controlled trial where male subjects with obesity and metabolic syndrome were randomized to receive amoxicillin or vancomycin for 7 days.[170] The vancomycin group had impaired insulin sensitivity as well as bile acid dehydroxylation with marked changes in gut microbiota composition (decreased Gram-positive bacteria and a simultaneous increase in Gram-negatives).[170] The authors proposed that a reduction in secondary bile acids in the vancomycin group was driven by a lack of Gram-positive bacteria, which are instrumental for production of secondary bile acids.[170] Interestingly no negative impact on these parameters was reported in the amoxicillin group, showing the importance of the type of antibiotic administered. Nevertheless, another parameter that should be investigated is the abundance of antibiotic resistance genes after antibiotic treatment. In one study, 28 samples per child were collected in the first 3 years of life to determine the impact of antibiotic treatments on the microbial diversity early in life and the presence of antibiotic resistance genes in children.[171] One group of infants received 9 to 15 treatments of antibiotics in the first 3 years of life whereas the control group received no antibiotic treatment. Results showed that children receiving antibiotics had less diverse microbiota with lower microbial stability especially around the time of the antibiotic exposure. Furthermore, antibiotic resistance genes rose rapidly during the antibiotic treatment and decreased when antibiotics were discontinued, which was correlated with relative abundances of certain bacteria carrying those genes[171]. For example, an increase in *Klebsiella pneumoniae* positively correlated with the β-lactamase resistance gene, which possesses the ability to

destroy the ring structure of β-lactam antibiotics (i.e. penicillins, cephalosporins and cephamycins) leading to antibiotic resistance.[171] Interestingly, almost 30% of children in the study harbored antibiotic resistance genes even before they were exposed to antibiotics possibly because of the environment (i.e. maternal milk contains antimicrobial peptides) or gut microbiota mother-to-child vertical transmission during birth.[171]

Taken together, the establishment of the infant gut microbiota appears to profoundly influence the development and health of a child, therefore early life disruptions such as antibiotics should be avoided whenever possible. Given the current epidemic of childhood obesity and the high prevalence of infant antibiotic exposure, studies are needed to develop interventions that could be co-administered with antibiotics to reduce the chance for metabolic harm, especially when early life antibiotic exposure cannot be avoided.

2.13 Modulation of gut microbiota in obesity with diet

While our individual host genome does not change over time, many environmental and lifestyle factors can profoundly change our gut microbiome throughout our lives[172]. One of the characteristics of gut microbiota that make it such an opportune target for new obesity treatments is the relative ease by which it can be manipulated with dietary agents. Interestingly, some gut microbes can remember past diets and exhibit a so called hysteric pattern that reflects those prior diets[173]. For example, when mice were put on a chow diet between two bouts of high-fat lard-based diet, accelerated weight regain was seen after the second exposure to the Western diet[174]. The authors were able to identify a gut microbiome signature that persisted after successful dieting in the obese mice and contributed to faster weight regain upon re-exposure to the high-fat diet[174]. Experiments in so-called “humanized mice” (germ free mice colonized with human fecal samples) also provide similar evidence in that the dietary history of the human donor determines the response to the diet intervention in mice[175]. This effect is transmittable across generations. When humanized mice were exposed to low fiber diet, reduced microbial diversity/function was seen and the effects were transmitted to future generations[176]. Loss of microbial diversity was greater with each subsequent generation (four in total) with an additional loss of microbial fiber-degrading capacity[176]. Exposing

the fourth generation of mice to a high fiber diet could not correct the loss of microbial diversity and function. Recapturing this function could only be achieved through reintroduction of lost bacteria with fecal microbiota transplant from control mice[176]. After the fecal transplant and a switch to a high fiber diet, 110 taxa were restored and the differences between low-fiber and high-fiber diet group were no longer detectable[176]. These studies demonstrate the importance of high fiber diet in order to prevent the loss of microbial taxa and function seen with consumption of low fiber Western diet[177].

2.14 Prebiotics

In 1995 when Gibson and Roberfroid first defined prebiotics, only a few compounds fit the definition including short- and long chain β-fructans [fructo-oligosaccharides (FOS), and inulin], galacto-oligosaccharides (GOS) and lactulose[178]. The most recent definition of prebiotics is that they are a substrate that is selectively utilized by host microorganisms conferring a health benefit[23]. Changes in the definition from its inception have enabled more compounds such as resistant starches, pectin, arabinoxylan, whole grains and non-carbohydrate compounds (polyphenols) to be considered as candidate or confirmed prebiotics[23,179]. Interestingly, not all dietary fibers can be classified as prebiotics as consumption of prebiotics must result in a health benefit for the host[179]. For example soluble dextrin fibers from corn failed to be classified as a prebiotic even though microbial changes in the gut were detected along with a lower secretion of pro-inflammatory and immunoregulatory cytokines, no improvement in histological colonic inflammation was seen[180]. It might be that the dose administered was too low to improve health as a dose-dependent effect of prebiotics on disease risk has been described with higher doses displaying more health benefits[181].

Intake of prebiotics has been associated with improvements in metabolic health that have included lower body weight and fat mass, improved glucose control, reduced inflammation and an increase in health promoting bacteria[23,24]. For example, in infants, breast milk is a rich source of human milk oligosaccharides (candidate prebiotics) that stimulate the growth of commensal bacteria (*Bifidobacterium and Bacteroides* spp.) and restrict the adhesion of pathogens such as *E. coli*, *Campylobacter jejuni* and *Heliobacter pylori*[182]. Already in 1935, a report from Massachusetts General Hospital convincingly

showed the benefits of breast-feeding by analyzing 20,000 patients[183]. Breast-fed infants had lower incidence of morbidity and mortality, especially of enteric disease, otitis media and respiratory infection when compared to exclusively formula-fed infants[183]. It is plausible that the microbiota, at least in part, is involved in these improved infant outcomes.

Oligofructose is an inulin-type fructan composed of β-D-fructofuranoses attached by β(2→1) glycosidic bonds.[184] It is produced by partial enzymatic hydrolysis of its longer chain parent compound inulin and it consists of up to 10 fructose units with or without a glucose end group[185]. A related short-chain prebiotic is fructooligosaccharide which is enzymatically synthesized from sucrose. Inulin and oligofructose are found in more than 36,000 plant species including chicory roots, onion, bananas, garlic and wheat, although at relatively low concentrations except for chicory roots.[186] β linkages (glycosidic bonds) between fructose units prevent inulin and oligofructose from being digested by humans, but intestinal bacteria such as *Bifidobacterium* and *Lactobacillus* strains ferment these compounds.[187] During the fermentation process, SCFAs are produced which confer a health benefit on the host through several mechanisms.[188] More than 95% of SCFAs are generated in the colon (humans) or cecum (rodents) conferring local benefits (colonocyte function) and distant ones such as appetite control, modulation of blood lipids and immune system function.[188] The most common SCFA produced from the fermentation of oligofructose and inulin is acetate (mostly produced by bifidobacteria), but via cross-feeding, other SCFAs including butyrate (mostly produced by *Lachnospiraceae* and *Ruminococcaceae)* and proprionate (made by propionibacteria and *Bacteroidetes)* can be produced.[179]

2.15 Prebiotics and obesity risk

Several studies have reported a correlation between a low abundance of *Bifidobacterium* and obesity[16,17] along with an increased capacity of obesogenic gut microbiota to produce SCFAs[50,92,189], however, both have been shown to be reversible with a prebiotic approach. In a randomized, double-blind, placebo controlled trial involving 48 men and women with overweight or obesity, a reduction in body weight, improvement in satiety hormone profiles (peptide YY, ghrelin) and reduced food intake

was seen after a 12 week intervention with 21 g/day of oligofructose.[190] In contrast, a double-blind placebo controlled study involving 30 women with obesity, failed to detect an effect on body weight after prebiotic exposure for 3 months (16g/day, inulin/oligofructose 50/50 mix) but the authors did show that shifts in the gut microbiota were associated with other positive health outcomes.[191] For example, in the prebiotic group, increases in *Clostridium* clusters IV and XVI were observed which negatively correlated with plasma LPS levels, changes in fat mass and fasting glucose.[191] Furthermore, the prebiotic increased *Bifidobacterium* and *F. prausnitzii* levels, which negatively correlated with LPS, an endotoxin and pro-inflammatory molecule.[191] A recent systematic review of 27 publications found fair evidence that prebiotics and substances with prebiotic properties promote weight loss and improve metabolic and/or inflammatory biomarkers in patients with type 2 diabetes mellitus (T2DM).[192]

Oligofructose-enriched inulin provides a blend of long chain (inulin) and short chain (oligofructose) fructans that ferment at different rates in the distal gut; oligofructose being fermented more rapidly. Intervention studies exposing normal weight healthy adolescents to oligofructose-enriched inulin (8g/day) for 1 year[193] reported decreased body weight gain and fat mass. Similarly, children (7-12 years of age) administered 8g/day of oligofructose-enriched inulin for 16 weeks had reduced body fat[25] and improved appetite control[194] compared to a placebo. Prebiotic consumption normalized childhood weight gain, reduced total and trunk body fat, altered primary fecal bile acids and altered microbiota composition by increasing *Bifidobacterium* species[25].

Mechanistically, several animal studies have provided insight into prebiotic-mediated outcomes noted in human studies. One possible mechanism for reduced body weight in prebiotic-fed animals is increased SCFA production due to prebiotic fermentation. Even though SCFAs are a source of calories, they also play a beneficial role in body weight regulation by targeting several tissues and acting as signal transduction molecules[26]. SCFAs have been shown to modulate the expression of G-coupled receptors and that in turn resulted in reduced body weight, enhanced triglyceride hydrolysis, fatty acid oxidation and protection against diet-induced obesity[26]. In addition, prebiotic intake in rodents led to: increased numbers/activity of enteroendocrine L-cells responsible for the production of satiety hormones[27,28] and improved glucose

homeostasis; recovery of gut barrier function through increased *Bifidobacterium* spp.[28,29] and expression/activity of tight junction proteins with a subsequent decrease in circulatory LPS levels[29,30]; reduced hepatic accumulation of triglycerides and cholesterol[195,196]; and improved weight maintenance and weight loss[27,28]. A recent study by Rios et al. (2019) reported lower LPS levels and body fat percentage, improved insulin sensitivity, total cholesterol, plasma triglycerides and an increase the abundance of *Bifidobacterium* after prebiotic (oligofructose) was co-administered with a HFS diet.[197] The authors propose that an increase in *Bifidobacterium* improved gut permeability (seen by lower LPS levels), leading to lower systemic inflammation and consequently improving insulin sensitivity.[197] Thus, the positive effects of prebiotic use likely go beyond weight loss as all of the benefits described also participate in the improvement of overall host health.

2.16 Combined antibiotic and prebiotic exposure

To our knowledge, no studies have investigated whether or not promoting beneficial shifts in the gut microbiota with prebiotics could mitigate some of the detrimental metabolic effects of concomitant early life antibiotic exposure. One study did investigate the impact of early exposure (from postnatal day 5 to 15) to amoxicillin or one of three prebiotics (fructo-oligosaccharides vs. galacto-oligosaccharides/long-chain fructan mix vs. acidic oligosaccharides) on microbiota development into adulthood in Wistar rats[31]. The treatments were all administered separately and each had an acute effect on gut microbiota composition at postnatal day 14. By postnatal day 131, only one minor microbiota change persisted in the group fed the galacto-oligosaccharides/long-chain fructan mix. The study concluded that all interventions (amoxicillin and 3 prebiotic groups) induced acute changes during the period of administration, however, the study failed to address what health consequences (body weight/fat, insulin sensitivity, glucose tolerance) such neonatal disruption of microbiota may have later in life, even when gut microbiota changes are no longer detectable. Furthermore, whether or not co-administration of prebiotics with antibiotics could mitigate the obesity risk associated with early life antibiotic exposure has not been examined and was addressed directly in my PhD studies.

2.17 Conclusion

The environment determines bacterial growth; therefore it is not surprising that external factors such as diet influence our gut microbial composition/function. Diet has the potential to outweigh or in some cases counteract the effect of host genetics, immunity and early-life disruptors (antibiotics, C-section)[87] on the gut microbiota. While a high fiber diet is beneficial to the gut microbial community, a Western diet with an abundance of highly processed foods, low in fiber and rich in fat and sugar, is a major threat to our gut microbiota. This threat may not be strictly confined to the generation that consumes it but the resultant dysbiosis could perpetuate across multiple generations. Given the current epidemic of childhood obesity and the high prevalence of infant antibiotic exposure, further studies are necessary to develop strategies to protect the vulnerable early life microbiota against excessive disruption due to antibiotic exposure. Examining the risk of obesity following prenatal and intrapartum antibiotic exposure from human cohort data and the potential of prebiotics to mitigate obesity risk associated with early life exposure to antibiotics in rodents were the chief goals of this dissertation.

2.18 Research objectives and hypotheses

Overall thesis objective: To examine in a rodent model whether prebiotic co-administration with antibiotics reduces the negative metabolic outcomes caused by early life antibiotic exposure and investigate in a human birth cohort if antibiotic exposure during pregnancy and/or birth increases the risk of obesity in infants.

Objective 1:

Determine the potential for combined maternal prebiotic and antibiotic intake during pregnancy and lactation to reduce obesity risk in dams and their offspring.

Our goal was to examine the ability of prebiotic supplementation during pregnancy and lactation to reduce antibiotic-associated obesity risk in offspring through restoration of gut microbiota, improved glycaemia and satiety hormone secretion in metabolically challenged offspring.

Hypothesis: Adding prebiotic oligofructose to the diet of pregnant and lactating rats will mitigate antibiotic-induced obesity risk in the offspring.

Objective 2:

Determine the effect of combined postnatal prebiotic and antibiotic intake on obesity risk in rats.

Our goal was to determine if early postnatal prebiotic supplementation when co-administered with a broad spectrum antibiotic (azithromycin) can reduce obesity risk in rats through restoration of gut microbiota, improved glycaemia, satiety hormone secretion and hypothalamic/hepatic gene expression profiles in metabolically challenged offspring.

Hypothesis: Administering prebiotic oligofructose to young rats at the same time as antibiotic will reduce obesity risk and improve metabolic outcomes.

Objective 3:

To investigate the association between maternal antibiotic exposure during birth and/or during pregnancy and body mass index (BMI) *z* scores in children in the first three years of life.

Using data from the Alberta-based low risk pregnancy birth cohort (All Our Families), our goal was to confirm previous findings of increased obesity risk in infants with maternal exposure to antibiotics during pregnancy and to answer the novel question of whether intrapartum antibiotics increased risk of obesity in children.

Hypothesis: Maternal exposure to antibiotics during pregnancy and birth will be positively associated with infant BMI *z* scores at 1, 2 and 3 years of age.

CHAPTER THREE: PREBIOTICS PREVENT ANTIBIOTIC-INDUCED OBESITY RISK AND IMPROVE METABOLIC AND GUT MICROBIOTA PROFILES IN RAT DAMS AND OFFSPRING

3.2 Abstract

Background & Aims: Exposure to antibiotics during pregnancy/lactation increases obesity risk in offspring. Prebiotics reduce fat mass and improve metabolic health. Our aim was to examine if maternal prebiotic supplementation during pregnancy/lactation reduces antibiotic-associated obesity risk in offspring.

Methods: Ten-week-old female Sprague-Dawley rats (n=60) were mated and randomized to: 1) Control [CTR], 2) Antibiotic [ABT] (low dose penicillin (LDP)), 3) Prebiotic [PRE] (10% oligofructose (OFS) diet) or 4) Antibiotic+Prebiotic [ABT+PRE] (LDP+10%OFS diet) throughout the 3rd week of pregnancy and lactation. At 9 weeks of age, offspring were fed high fat/high sugar (HFS) diet for 8 weeks to unmask obesity risk. Dams and offspring underwent insulin and glucose tolerance tests and body composition measurements. Microbiota composition was assessed in fecal and cecal matter of dams and offspring.

Results: Dams given antibiotics alone had higher body weight, body fat and leptin during lactation than all other groups. At the end of lactation, PRE and ABT+PRE dams had lower hepatic triglycerides and greater *Bifidobacterium* in cecal matter. ABT offspring had increased early growth rates after weaning and were heavier after HFS diet challenge than other groups. This phenotype was preventable with prebiotics. Higher fat mass and liver triglycerides were seen in male ABT offspring. At weaning, male ABT offspring had lower *Lactobacillus* while PRE and ABT+PRE offspring had higher levels of *Bifidobacterium* spp. and *Collinsella*.

Conclusions: We show that antibiotic use during pregnancy/lactation impairs normal postpartum weight loss in dams. Co-administering prebiotics with antibiotics prevented obesity risk in offspring, likely in part through effects mediated by alterations in the maternal and offspring microbiota.

3.3. Introduction

According to the World Health Organization, worldwide obesity has more than tripled in the last 40 years[32]. A variety of contributors to obesity's initiation and maintenance that involve genetic and environmental factors have been described[34]. Given the gut microbiota's influence on energy harvest, inflammation and metabolic signalling, it is now considered an important factor contributing to obesity and its co-morbidities[36]. The earliest evidence of gut microbiota involvement in regulating body weight was the observation that germ-free (GF) mice are leaner compared to conventionally raised (CONV-R) mice, and transplantation of conventional gut microbiota into adult GF mice substantially increased their fat mass despite reduced food intake[77].

The gut microbiome (the gene pool of the gut microbiota) develops over the course of childhood and reaches its adult form at approximately three years of age in humans[39]. Several factors influence microbial colonization of the infant gut such as delivery mode, breastfeeding versus formula feeding and antibiotic treatment[52]. Antibiotics represent the most widely prescribed therapeutic agents[131]. Although antibiotics are life-saving drugs, early-life antibiotic exposure is associated with increased risk of developing obesity, asthma and neurodevelopmental disorders[198]. A study of 436 mother-child pairs reported an average 84% increased risk of obesity in children at seven years of age when their mothers received antibiotics in the second or third trimester of pregnancy[12]. Likewise, a population-based study in Denmark showed that women who received amoxicillin during pregnancy tended to give birth to infants with higher birth weights[158]. The weight gain observed after early-life antibiotic treatment is more pronounced in boys/males[20,130,141,155,165] and appears to be a consequence of reduced abundance of potentially metabolically protective bacteria (such as *Lactobacillus* and *Allobaculum*), increased availability of microbiota-derived energy, altered hepatic metabolic signaling, and/or intestinal immunity[21].

The gut microbiota is profoundly influenced by diet including prebiotics - substrates that are selectively utilized by host microorganisms conferring a health benefit[188]. Prebiotic intake, particularly inulin and oligofructose, is associated with improvements in metabolic health resulting in lower body weight and fat mass, improved glucose control, reduced inflammation and an increase in health promoting bacteria[188].

Several studies have shown a correlation between low levels of *Bifidobacterium* spp. and obesity[16,17]. Prebiotics, because of their bifidogenic effect[188], have the potential to reverse *Bifidobacterium* deficiencies and mitigate obesity risk.

Given the high prevalence of maternal antibiotic exposure, with >50% of pregnant women in the USA receiving antibiotics during pregnancy[39], strategies are critically needed to reduce the risk of metabolic harm to offspring, especially when antibiotic exposure cannot be avoided. Studies in humans[199,200] and animals[130] show that microbiota-dependent mechanisms drive increased obesity risk in offspring when mothers received antibiotics during pregnancy[130]. However, no studies to date have examined the potential of prebiotic co-administration to reverse the metabolic disruptions caused by gestational antibiotic treatment. Therefore our objective was to investigate if concomitant administration of oligofructose could attenuate antibiotic-induced obesity risk in dams and their offspring.

3.4 Materials and Methods

3.4.1 Animals and Diets

A total of 70 Sprague Dawley rats (10wk old, n=60 females, n=10 males) were obtained from Charles River Laboratories (Saint Constant, QC, Canada). Two animals from the same dam were housed together a 12 h light–dark cycle in a temperature and humidity controlled room. After two weeks of acclimatization, dams were mated and randomized into one of four groups beginning at the third week of pregnancy: 1) Control [CTR], 2) Antibiotic [ABT] (low dose penicillin G (LDP); 1 μg/g; Sigma Aldrich, Oakville, ON, Canada), 3) Prebiotic [PRE] (10% oligofructose (OFS) diet; 10% wt/wt, Orafti P95, Beneo) or 4) Antibiotic+Prebiotic [ABT+PRE] (LDP+10%OFS diet). Dams received LDP via drinking water throughout the third week of pregnancy and all of lactation as described previously[130,141]. The 10% OFS dose was selected based on previous rodent experiments showing reductions in fat mass[201,202] and increases in *Bifidobacterium* favoring a lean phenotype[89,203]. The amount of penicillin added to the drinking water was calculated based on water consumption and body weight. The dose administered (1 μg/g) was in the mid-range approved by US Food and Drug

Administration (FDA) for use in agriculture[130,141]. LDP was selected based on previous evidence by the MJ Blaser group that subtherapeutic doses of penicillin in young mice was a potent inducer of obesity[130,204]. Water containers were changed twice weekly to supply fresh antibiotics[141]. Dams consumed control diet (AIN-93G, Dyets Inc., Bethlehem, PA, USA) or 10% OFS diet throughout the third week of pregnancy and lactation.

Within 24 hours of birth, litters were culled to ten pups (n=5 M/F each) and extra pups were cross-fostered within treatments to dams with less than ten pups. Pups were weaned at three weeks of age onto control diet (AIN-93G) until nine weeks of age, when rats received a high-fat/high sucrose diet (HFS) (diet #102412; Dyets, Bethlehem, PA, USA) until 17 weeks of age, which served as a metabolic challenge to unmask programmed obesity risk/protection[130]. A separate lean control group of male (n=10) and female (n=10) offspring derived from the CTR dams were maintained solely on control AIN-93 diet (no HFS exposure) to serve as a reference to normal development. Ethical and study protocol approval was granted by the University of Calgary Animal Care Committee (Protocol #AC15-0079) and conformed to the *Guide to the Care and Use of Laboratory Animals*.

3.4.2 Body Weight and Composition

Maternal and offspring body weight was measured weekly throughout the study. Pups were also weighed daily immediately post-weaning (day 21) until day 31 to assess early growth rates as described previously[130]. At weaning (dams) and at 17 weeks of age (offspring), rats were lightly anaesthetized with isoflurane and body composition was measured via dual energy x-ray absorptiometry (DXA) scan with software for small animals (Hologic ODR 4500; Hologic Inc., Bedford, MA, USA).

3.4.3 Food and Fluid Intake

Dams food (weight) and fluid (volume) intake was measured every week throughout pregnancy and lactation for five consecutive days each week. Pups food and water consumption was recorded at weeks 3, 8, 10 and 17 for five consecutive days at each time point. Since two animals were housed together, the average daily food

consumption per animal was calculated by dividing cage consumption by two to acquire food intake/animal/day.

3.4.4 Oral Glucose and Insulin Tolerance Tests

Dams underwent an oral glucose tolerance test (OGTT) on the last day of lactation and the offspring underwent an OGTT at nine and 16 weeks of age. OGTTs were performed according to our previous work with a 2 mg/kg glucose load[205]. Insulin tolerance tests were performed in the offspring at nine and 15 weeks of age following a 6-hour fast and with a 0.75U/kg insulin load. Blood glucose levels during OGTT and ITT were measured at 0 (baseline), 15, 30, 60, 90 and 120 minutes. Blood glucose was determined immediately using OneTouch Verio Blood Glucose Meter (OneTouch®).

3.4.5 Tissue and Blood Collection

Animals were euthanized by overanesthetization and aortic cut. Fasted blood was collected from the portal vein into chilled tubes containing diprotinin-A (0.034 mg/ml blood; MP Biomedicals, Irvine, CA, USA), Sigma protease inhibitor (1 mg/ml blood; Sigma Aldrich, Oakville, ON, Canada) and Roche Pefabloc (1mg/ml of blood; Roche, Mississauga, ON, Canada). Tissue samples from the cecum and liver and cecal luminal contents were collected from pups (17 weeks old) and dams (end of lactation) and snap frozen in liquid nitrogen. All tissues were stored in -80°C.

3.4.6 Serum Hormones and Hepatic Triglyceride Analysis

Serum GLP-1, insulin and leptin were analyzed with a Luminex rat metabolic hormone 3-plex assay (Eve Technologies, Calgary, AB, Canada). A portion of the left liver lobe of each animal (25 mg) was used to quantify tissue triglyceride (TG) content using a Triglycerides LiquiColor™ Test Mono reagent set (Stanbio™, Boerne, TX, USA) according to our previous work[89].

3.4.7 Fecal Collection and qPCR

Maternal fecal samples were collected on gestation day 7 (baseline prior to treatments), lactation days 1 and 7. Fecal samples from the offspring were collected at week 3 (immediately after weaning), week 8 (before a HFS diet challenge) and week 17 of life (end of the study and HFS challenge). Using ~ 250mg of fecal matter, total

bacterial DNA was extracted using a FastDNA Spin Kit for feces (MP Biomedicals) with bead beating and quantified using Nanodrop 2000 (Thermo Fisher Scientific Life Sciences, Waltham, MA, USA). Quantitative PCR (qPCR) was carried out for select bacterial groups as previously described[206]. Results are presented as relative abundance (%) of total bacterial gene copies measured. The group-specific primers are described in Table 3.1.

Table 3.1 Gut microbial group specific primers and genomic DNA standards for qPCR.

Microbial Group	Primer Sequence, 5'-3' (Forward, F and Reverse, R)	Genomic DNA Standard	Reference
Firmicutes			
Clostridium coccoides (cluster XIV)	F: ACTCCTACGGGAGGCAGC R: GCTTCTTAGTCARGTACCG	*Ruminococcus productus*	Amann, Krumholz, & Stahl, 1990; Franks et al., 1998
Clostridium leptum (cluster IV)	F: GCACAAGCAGTGGAGT R: CTTCCTCCGTTTGTCAA	*Clostridium leptum*	Matsuki, Watanabe, Fujimoto, Takada, & Tanaka, 2004
Clostridium group (cluster I)	F: ATGCAAGTCGAGCGAKG R: TATGCGGTATTAATCTYCCTTT	*Clostridium perfringens*	Rinttila, Kassinen, Malinen, Krogius, & Palva, 2004
Clostridium group (cluster XI)	F: ACGCTACTTGAGGAGGA R: GAGCCGTAGCCTTTCACT	*Clostridium difficile*	Song, Liu, & Finegold, 2004
Faecalibacterium prausnitzii	F: AACCTTACCAAGTCTTGACATC R: TTGCGTAGTAACTGACCATAAG	*F. prausnitzii*	Beacon Designer 3.0
Lactobacillus	F: GAGGCAGCAGTAGGGAATCTTC R: GGCCAGTTACTACCTCTATCCTTCTTC	*Lactobacillus jensonii*	Delroisse et al., 2008
Roseburia	F: TACTGCATTGGAAACTGTCG R: CGGCACCGAAGAGCAAT	*Roseburia hominis*	Larsen et al., 2010
Bacteroidetes			
Bacteroides /Prevotella	F: TCCTACGGGAGGCAGCAGT R: CAATCGGAGTTCTTCGTG	*Bacteroides thetaiotaomicron*	Bernhard & Field, 2000; Nadkarni, Martin, Jacques, & Hunter, 2002
Actinobacteria			

Bifidobacterium	F: CGCGTCYGGTGTGAAAG R: CCCCACATCCAGCATCCA	*B. adolescentis*	Delroisse et al., 2008
Collinsella aerofaciens	F: CCCGACGGGAGGGGAT R: CTTCTGCAGGTACAGTCTTGAC	*Collinsella aerofaciens*	Desai et al., 2016
Archaea Methanobrevibacter	F: CTCACCGTCAGAATCGTTCCAGTC R: ACTTGAGATCGGGAGAGGTTAGAGG	*M. smithii*	Bomhof et al., 2014
Proteobacteria Enterobacteriaceae	F: CATTGACGTTACCCGCAGAAGC R: CTCTACGAGACTCAAGCTTGC	*Escherichia coli*	Bartosch, Fite, Macfarlane, & McMurdo, 2004
Verrucomicrobia Akkermansia muciniphila	F: TCTTCGGAGGCGTTACACAG R: AGTTGATCTGGGCAGTCTCG	*Akkermansia muciniphila*	Beacon Designer 3.0

3.4.8 Cecal 16S rRNA Illumina Sequencing

Cecal DNA was extracted as described above (Fecal Collection and qPCR), quantified (PicoGreen kit, Invitrogen) and diluted to 20 ng/μl for sequencing. Microbial sequencing was performed on the MiSeq Illumina platform at the Centre for Health Genomics and Informatics (University of Calgary). The V3 and V4 regions of the 16S rRNA gene were amplified with a protocol involving a two-step, tailed PCR approach that generated ready-to-pool amplicon libraries as described previously[206]. The pooled and indexed library set was denatured, diluted, and sequenced in paired-end modus on an Illumina MiSeq (Illumina Inc., San Diego, USA). Sequences were checked for quality, trimmed to 250 nts, merged, checked for chimeras and filtered using the FASTX-toolkit[25]. Operational Taxonomic Units (OTUs) with 98% homology were identified using USEARCH. Sequences were classified from phylum to genus level using the Ribosomal Database Project MultiClassifier. All taxonomic data was calculated as proportions of sequences based on the total number of sequences for each sample. To reduce biases introduced by DNA amplification (i.e. PCR) and by sequencing errors, we excluded any OTU that was found less than two times in the community matrix. This resulted in 1041 OTUs. We used the phyloseq[207] and vegan[208] packages for R (R Development Core Team; http://www.R-project.org) to analyse the variation in gut bacterial alpha- and beta-diversity and generate the plots.

3.4.9 Fecal Microbiota Transplant (FMT)

Cecal matter collected from ABT, PRE, ABT+PRE and CTR offspring at the end of the study (Wk 17) and stored at -80°C was subsequently pooled and mixed with 5ml of sterile phosphate buffered saline solution in an anaerobic chamber to create a homogenous solution. A 400µl volume of the PBS-cecal matter solution was gavaged into germ-free mice (n=10-14/group). Body weight was measured at baseline, three, seven, ten, 14, 17 and 21-days following gavage. At 22-days post gavage, a DXA scan was performed and and then food removed for 5 hours prior to euthanasia to obtain a fasted blood sample. Fasting blood glucose levels were determined using OneTouch Verio Blood Glucose Meter (OneTouch®) and fasting insulin was measured using ELISA (Millipore, Etobicoke, ON, Canada).

3.4.10 Statistical Analysis-16S rRNA Illumina Sequencing

To estimate gut bacterial alpha-diversity, we measured Chao1 and Shannon indices. A non-parametric Kruskal-Wallis test followed by a Dunn's *post hoc* was used to test for significant differences in alpha-diversity between treatments for each group separately (dams, male offspring, female offspring). To account for potential heteroskedasticity in community beta-diversity dispersion and to avoid loss of information through rarefaction[209], we performed a variance stabilizing transformation[209,210] prior to any statistical tests. Changes in gut bacterial community structure (beta-diversity) were assessed statistically using Permutational Multivariate Analysis Of Variance (PERMANOVA) and visualized using Principal Component Analysis (PCoA) based on Bray-Curtis dissimilarities. To explore further the changes in taxonomical community structure, we tested for significant changes in relative abundance using non-parametric Kruskal-Wallis test. If the test was significant, pairwise Wilcoxon rank sum test was performed with a Benjamin-Holmes False Discovery Rate (FDR) correction for multiple testing to describe differences between groups.

3.4.11 Statistical Analysis – Biological and qPCR Outcomes

All data is presented as mean ± standard error of the mean (SEM). Boxplots were made to identify outliers and normality was assessed using the Shapiro-Wilk test. If the data was normally distributed, one-way ANOVA with Tukey's *post hoc* tests was used.

For longitudinal and timed data, a linear mixed-model for repeated measures, followed by an ANOVA with Tukey's *post hoc* was used. The lean reference group serves as a benchmark of normal weight gain and development and therefore was not included in the statistical analysis after the obesity induction period. In all tests, significance was set at $p<0.05$. Statistical analyses were performed using SPSS version 24.0.0.2 software (SPSS, Inc., Chicago, IL, USA).

3.5 Results

3.5.1 Maternal Antibiotic/Prebiotic Exposure Regulates Post-Partum Weight Loss and Metabolism

Antibiotics administered from gestation (G) d14 to lactation (L) d21increased maternal body weight in the third week of pregnancy and animals retained significantly more weight throughout lactation ($p<0.0001$; Figure 3.1A); both outcomes were attenuated with prebiotic co-administration. ABT dams had greater fat ($p<0.0001$; Figure 3.1F) but not lean mass ($p=0.278$; Figure 3.1E) and higher liver triglyceride levels compared to PRE and ATB+PRE ($p=0.003$; Figure 3.1G). Higher fasting leptin levels were detected in the ABT group at euthanasia ($p<0.0001$; Figure 3.1J), which is consistent with greater fat mass in the ABT group (Figure 3.1I). Dams consuming prebiotics had reduced caloric intake immediately upon exposure to the diet (Gwk3, Figure 3.1B) and had increased serum levels of the satiety hormone GLP-1 at the end of lactation ($p<0.0001$; Figure 3.1H). Prebiotics improved glucose tolerance at 30 minutes during the OGTT ($p=0.005$; Figure 3.1C) but had no impact on insulin sensitivity ($p=0.489$; Figure 3.1D) and fasting insulin levels at euthanasia ($p=0.085$; Figure 3.1J).

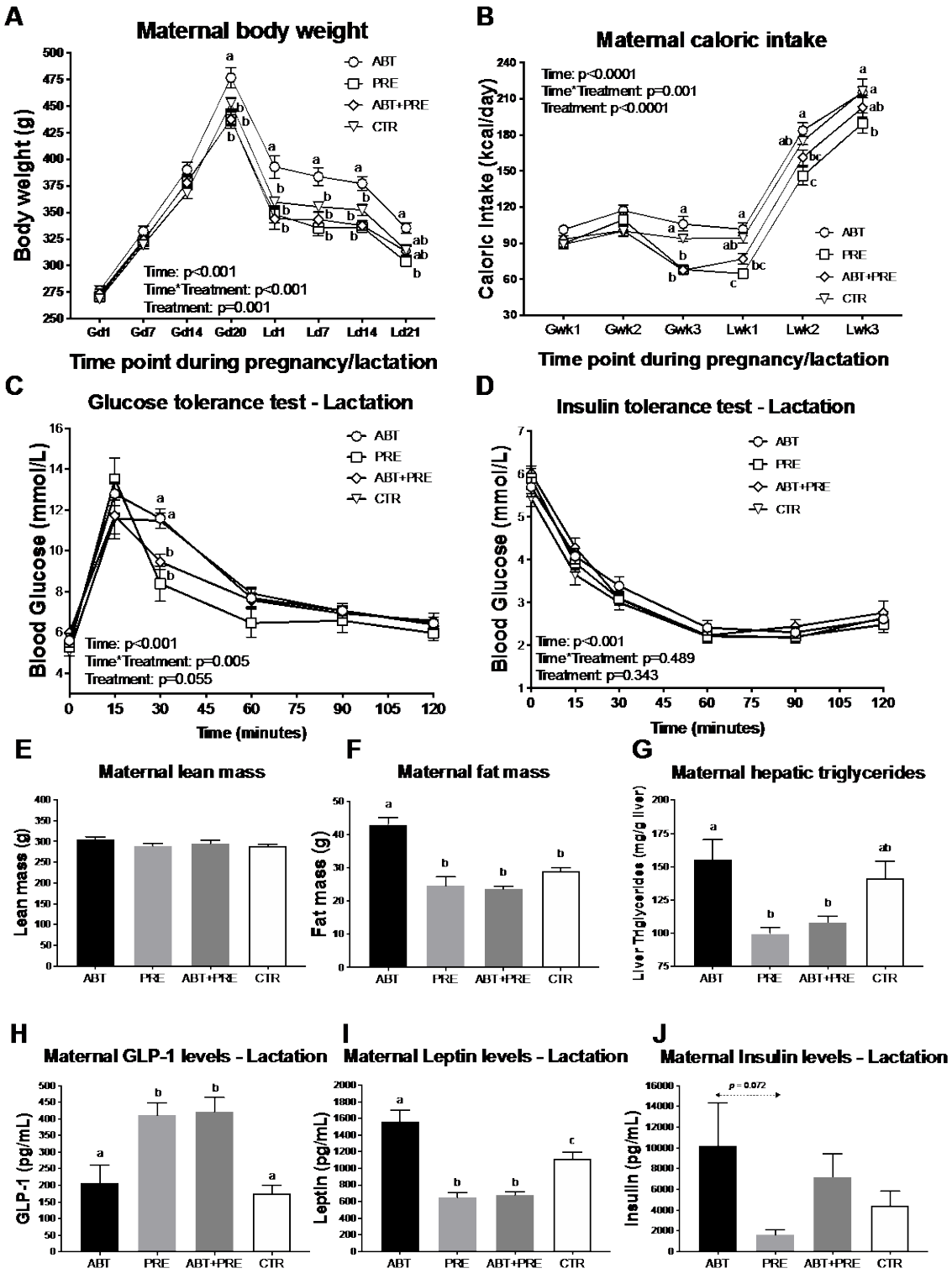

A
Maternal body weight
Body weight (g)
ABT
PRE
ABT+PRE
CTR
Time: p<0.001
Time*Treatment: p<0.001
Treatment: p=0.001
Gd1
Gd7
Gd14
Gd20
Ld1
Ld7
Ld14
Ld21
Time point during pregnancy/lactation
B
Maternal caloric intake
Caloric Intake (kcal/day)
Time: p<0.0001
Time*Treatment: p=0.001
Treatment: p<0.0001
Gwk1
Gwk2
Gwk3
Lwk1
Lwk2
Lwk3
Time point during pregnancy/lactation
C
Glucose tolerance test - Lactation
Blood Glucose (mmol/L)
Time: p<0.001
Time*Treatment: p=0.005
Treatment: p=0.055
Time (minutes)
D
Insulin tolerance test - Lactation
Blood Glucose (mmol/L)
Time: p<0.001
Time*Treatment: p=0.489
Treatment: p=0.343
Time (minutes)
E
Maternal lean mass
Lean mass (g)
F
Maternal fat mass
Fat mass (g)
G
Maternal hepatic triglycerides
Liver Triglycerides (mg/g liver)
H
Maternal GLP-1 levels - Lactation
GLP-1 (pg/mL)
I
Maternal Leptin levels - Lactation
Leptin (pg/mL)
J
Maternal Insulin levels - Lactation
Insulin (pg/mL)
p = 0.072

Figure 3.1 Maternal antibiotic/prebiotic exposure regulates post-partum weight loss and metabolism.

(A) Body weight of dams during pregnancy and lactation (n=9-12 rats/group). (B) Maternal caloric intake (kcal) calculated as average energy intake over 5 days measured for 6 consecutive weeks during pregnancy and lactation (n=9-12 rats/group). (C and D) Glucose response in dams at the end of lactation measured by OGTT (C) and ITT (D) (n=8-10 rats/group). Lean (E) and fat mass (F) of dams at the end of lactation (n=9-11 rats/group). (G) Liver triglyceride levels in dams at the end of lactation (n=7-9 rats/group). Portal vein GLP-1 (H), leptin (I) and insulin (J) measured at euthanasia (n=8-12 rats/group). Results are shown as mean ± SEM. Labelled means without a common superscript letter differ ($p < 0.05$). ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; CTR, control; Gd, gestation day; Ld, lactation day; Gw, gestation week; Lw, lactation week.

3.5.2 Microbial Profiles are Influenced by Maternal Antibiotic/Prebiotic Exposure During Pregnancy and Lactation

To gain insight into the temporal changes in the microbiota, fecal matter (Figure 3.2E-J and Figure 3.3A-G) was collected throughout pregnancy and lactation. Select microbial groups were assessed with qPCR to quantitatively measure the impact of antibiotics/prebiotics on the fecal microbiota of dams. At birth (Ld1) higher levels of *Enterobacteriaceae* (Figure 3.2F) and lower levels of *Lactobacillus* spp. (Figure 3.2E) were observed in ABT dams; both were normalized with a prebiotic approach. Prebiotic groups (PRE, ABT+PRE) had higher levels of *Bifidobacterium* spp. (Figure 3.2G) and *Collinsella aerofaciens* (Figure 3.2H) on Ld7 compared to ABT and CTR groups. In addition, lower levels of *Clostridium leptum* (Figure 3.2I) and *Clostridium coccoides* (Figure 3.2J) were seen in PRE and ABT+PRE compared to ABT and CTR.

Cecal content was collected from dams at euthanasia when pups were weaned and the microbiota profiled using Illumina 16S rRNA sequencing (Figure 3.2A-D, Table 3.2). Antibiotic treatment led to a greater relative abundance of *Verrucomicrobiaceae* (Figure 3.2A) in ABT dams only, which was due to greater abundance of *Akkermansia muciniphila* (Table 3.2, OTUs). Prebiotic-treated dams (PRE, ABT+PRE) had greater abundance of *Bifidobacteriaceae* (Figure 3.2A and Table 3.2) and *Collinsella aerofaciens*

(Table 3.2). Correlation analysis between biological outcomes and the 27 most abundant cecal OTUs showed strong correlations between OTUs and GLP-1/leptin/cecum size (Figure 3.4). Specifically, *Bifidobacterium choerinum* (OTU12) was negatively correlated with leptin and positively with GLP-1 and cecum size (Figure 3.4). Similarly, *Collinsella aerofaciens* (OTU18) was negatively correlated with body weight and positively with GLP-1 and cecum size (Figure 3.4). Prebiotic diet had a strong impact on alpha- and beta-diversity (Figure 3.2B-D). Dams on the prebiotic diet clustered together when beta-diversity was visualized with PCoA (Figure 3.2B). PERMANOVA analysis showed that the experimental treatments explained 67.5% of the variation in bacterial community structure (Figure 3.2B). PRE and ABT+PRE groups had lower alpha-diversity assessed with Chao1 (Figure 3.2C) and Shannon index (Figure 3.2D), suggesting that this dietary intervention selects for a reduced number of taxa that explains the majority of the bacterial community.

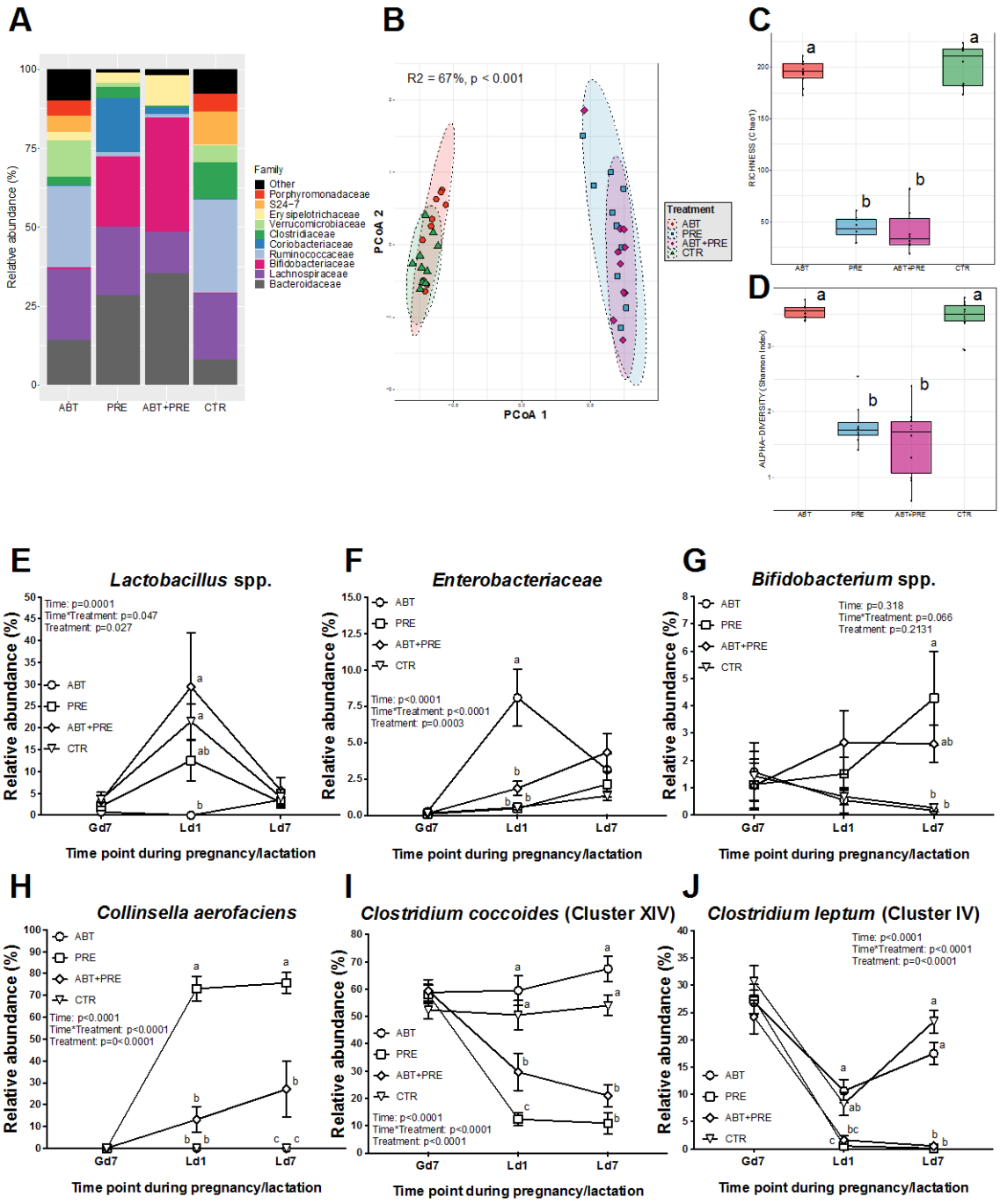
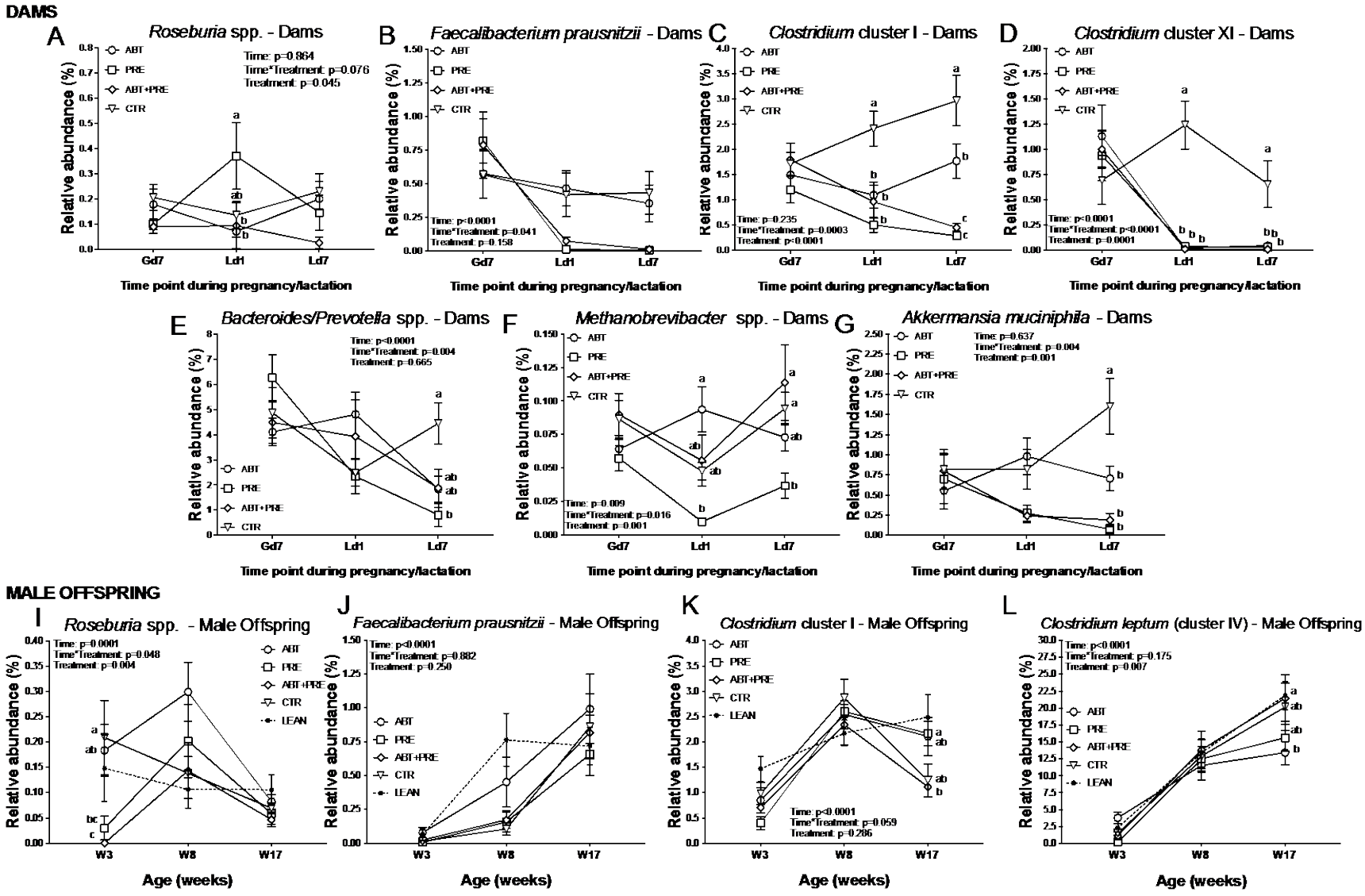


Figure 3.2 Microbial profiles are influenced by maternal antibiotic/prebiotic exposure during pregnancy and lactation.

Cecal matter was collected at euthanasia and sequenced using 16S rRNA Illumina sequencing. (A) Relative abundance of the ten most abundant bacterial families relative to prebiotic/antibiotic exposure (n=10 rats/group). (B) Principal-component analysis

(PCoA) ordination of variation in beta-diversity of gut bacterial communities based on Bray-Curtis dissimilarities among cecal samples (n=10 rats/group). (C) Chao1 estimated richness and (D) Shannon Index display differences in alpha-diversity between groups (n=10 rats/group). Fecal samples were collected repeatedly (Gd7, Ld1 and 7) and quantified with qPCR. Results are presented as relative abundance (%) for (E) *Lactobacillus* spp., (F) *Enterobacteriaceae*, (G) *Bifidobacterium* spp., (H) *Collinsella aerofaciens*, (I) *Clostridium coccoides* (Cluster XIV) and (J) *Clostridium leptum* (Cluster IV) (n=8-10 rats/group). Results are shown as mean ± SEM. Labelled means without a common superscript letter differ ($p < 0.05$). ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; CTR, control; Gd, gestation day; Ld, lactation day.


DAMS
A
Roseburia spp. - Dams
Time: p=0.864
Time*Treatment: p=0.076
Treatment: p=0.045
B
Faecalibacterium prausnitzii - Dams
Time: p<0.0001
Time*Treatment: p=0.041
Treatment: p=0.158
C
Clostridium cluster I - Dams
Time: p=0.235
Time*Treatment: p=0.0003
Treatment: p<0.0001
D
Clostridium cluster XI - Dams
Time: p<0.0001
Time*Treatment: p<0.0001
Treatment: p=0.0001
E
Bacteroides/Prevotella spp. - Dams
Time: p<0.0001
Time*Treatment: p=0.004
Treatment: p=0.665
F
Methanobrevibacter spp. - Dams
Time: p=0.009
Time*Treatment: p=0.016
Treatment: p=0.001
G
Akkermansia muciniphila - Dams
Time: p=0.637
Time*Treatment: p=0.004
Treatment: p=0.001
ABT
PRE
ABT+PRE
CTR
Relative abundance (%)
Gd7
Ld1
Ld7
Time point during pregnancy/lactation
MALE OFFSPRING
I
Roseburia spp. - Male Offspring
Time: p=0.0001
Time*Treatment: p=0.048
Treatment: p=0.004
J
Faecalibacterium prausnitzii - Male Offspring
Time: p<0.0001
Time*Treatment: p=0.882
Treatment: p=0.250
K
Clostridium cluster I - Male Offspring
Time: p<0.0001
Time*Treatment: p=0.059
Treatment: p=0.286
L
Clostridium leptum (cluster IV) - Male Offspring
Time: p<0.0001
Time*Treatment: p=0.175
Treatment: p=0.007
LEAN
W3
W8
W17
Age (weeks)

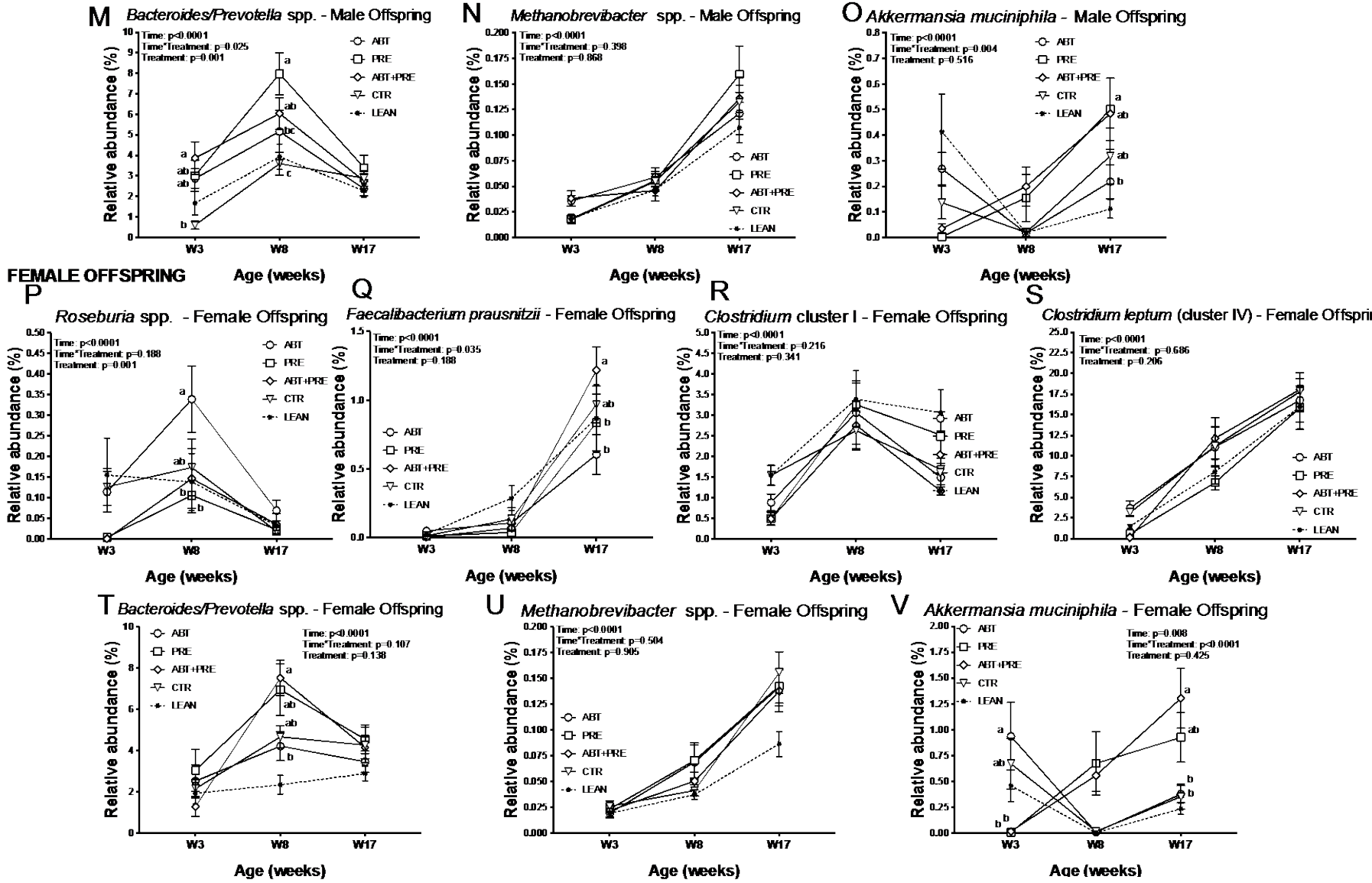

M
Bacteroides/Prevotella spp. - Male Offspring
N
Methanobrevibacter spp. - Male Offspring
O
Akkermansia muciniphila - Male Offspring
FEMALE OFFSPRING
P
Roseburia spp. - Female Offspring
Q
Faecalibacterium prausnitzii - Female Offspring
R
Clostridium cluster I - Female Offspring
S
Clostridium leptum (cluster IV) - Female Offspring
T
Bacteroides/Prevotella spp. - Female Offspring
U
Methanobrevibacter spp. - Female Offspring
V
Akkermansia muciniphila - Female Offspring
Relative abundance (%)
Age (weeks)
W3
W8
W17
ABT
PRE
ABT+PRE
CTR
LEAN

Figure 3.3 Maternal antibiotic/prebiotic exposure during pregnancy and lactation impacts microbial profiles of dams, male and female offspring.

Maternal fecal samples were collected repeatedly (gestation day 7 - baseline, lactation days 1 and 7) and microbial groups quantified using qPCR (A-G). Results are presented as relative abundance (%) for (A) *Roseburia* spp., (B) *Faecalibacterium prausnitzii*, (C) *Clostridium* cluster I, (D) *Clostridium* cluster XI, (E) *Bacteroides/Prevotella*, (F) *Methanobrevibacter* spp. and (G) *Akkermansia muciniphila* (n=8-10 rats/group). Fecal samples in male (I-O) and female (P-V) offspring were collected repeatedly (W3 - after weaning; W8 - before HFS challenge and W17 – end of study) and microbial groups quantified using qPCR. Results are presented as relative abundance (%) for (I,P) *Roseburia* spp., (J,Q) *Faecalibacterium prausnitzii*, (K,R) *Clostridium* cluster I, (L,S) *Clostridium* cluster IV, (M,T) *Bacteroides/Prevotella*, (N,U) *Methanobrevibacter* spp. and (O,V) *Akkermansia muciniphila* (n=7-10 rats/group). Results are shown as mean ± SEM. Labelled means without a common superscript letter differ ($p < 0.05$). ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; CTR, control; LEAN, lean control; Gd, gestation day; Ld, lactation day.

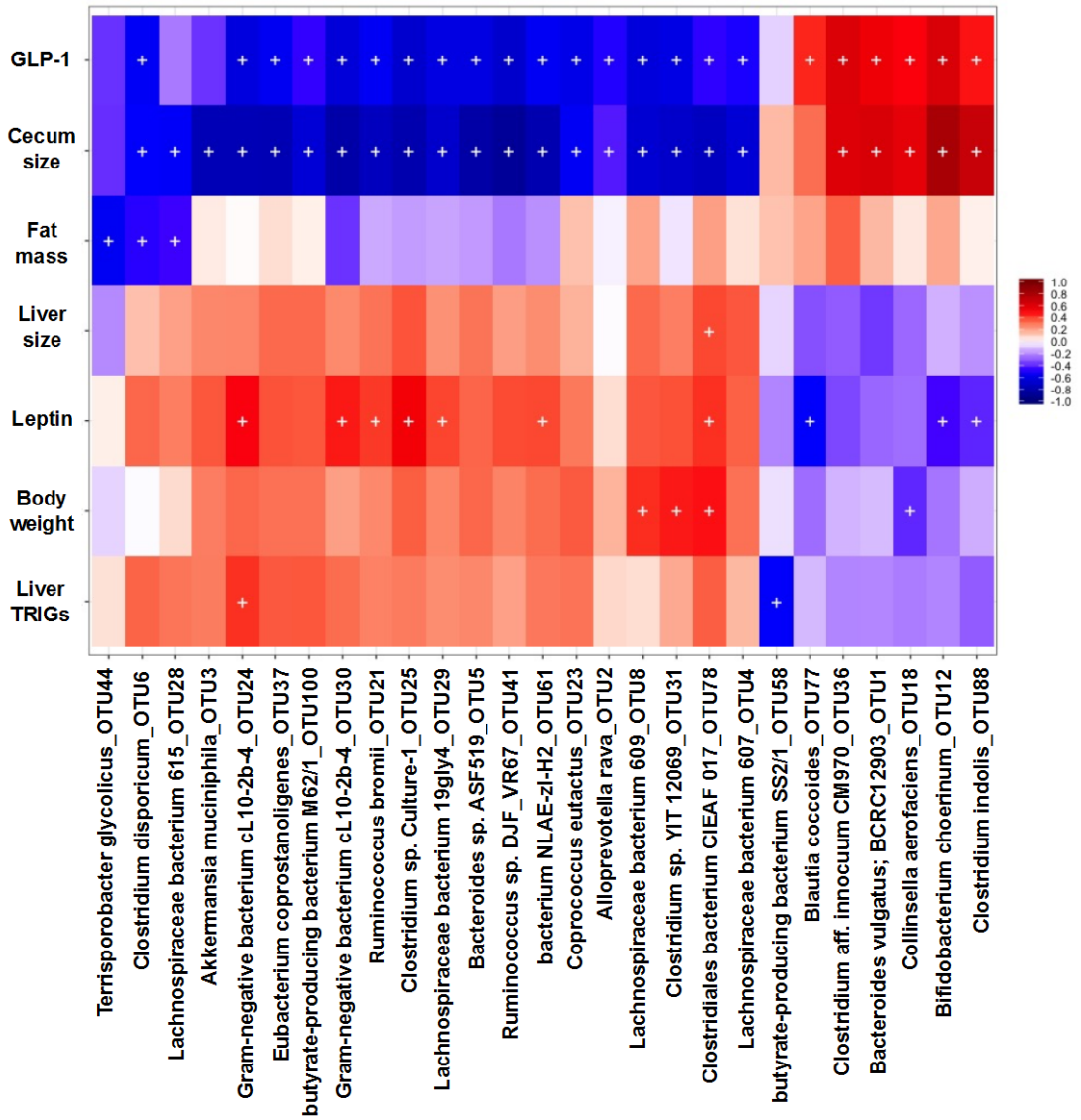


Figure 3.4 Heat map of the Spearman rank correlations between metabolic/phenotypic profiles and the 27 most abundant cecal OTUs in dams. Correlations were performed on the measurements performed at sacrifice (end of study). Colors denote positive (red) and negative (blue) correlation values. Significant correlations are denoted with a plus sign ($p<0.05$; FDR). GLP-1 – glucagon-like peptide 1; Liver TRIGs – triglycerides

Table 3.2 Relative Abundances of Bacterial Taxa in Ceca of Dams at the End of Lactation Using Illumina 16S rRNA Gene Sequencing.

[P value (adj. val.), FDR significance set at 0.05; n=10 rats/group. OTU, operational taxonomic units.]

	Control	Antibiotic	Prebiotic	Antibiotic+Prebiotic
Phyla				
Firmicutes	73.9 ± 1.9^{c}	62.9 ± 2.5^{a}	32.9 ± 3.6^{b}	28.4 ± 4.4^{b}
Verrucomicrobia	3.677 ± 0.834^{b}	8.820 ± 1.621^{a}	1.218 ± 1.185^{c}	0.009 ± 0.005^{c}
Proteobacteria	0.532 ± 0.108^{a,b}	1.165 ± 0.321^{a}	0.253 ± 0.047^{b}	0.448 ± 0.087^{a,b}
Actinobacteria	0.566 ± 0.126^{a}	0.964 ± 0.153^{a}	36.911 ± 4.176^{c}	36.195 ± 6.968^{c}
Deferribacteres	0.016 ± 0.005^{a}	0.016 ± 0.007^{a}	0 ± 0^{b}	0 ± 0^{b}
Tenericutes	0.077 ± 0.040^{a}	0.129 ± 0.063^{a}	0 ± 0^{b}	0 ± 0^{b}
unclassified_Proteobacteria	0.147 ± 0.043^{a}	0.134 ± 0.052^{a}	0.002 ± 0.002^{b}	0 ± 0^{b}
Class				
Clostridia	72.4 ± 1.9^{a}	60.0 ± 2.7^{b}	28.0 ± 3.5^{c}	15.0 ±4.8^{d}
Erysipelotrichia	0.43 ± 0.04^{b}	2.08 ± 0.33^{a}	3.12 ± 1.56^{a,b,c}	9.22 ± 2.32^{c}
Verrucomicrobiae	3.677 ± 0.834^{b}	8.820 ± 1.621^{a}	1.218 ± 1.185^{c}	0.009 ± 0.005^{c}
Alphaproteobacteria	0.341 ± 0.089^{a}	0.951 ± 0.272^{a}	0.002 ± 0.001^{b}	0.010 ± 0.003^{c}
Gammaproteobacteria	0.005 ± 0.002^{b}	0.031 ± 0.009^{a}	0.145 ± 0.054^{a,c}	0.339 ± 0.088^{c}
Actinobacteria	0.566 ± 0.126^{a}	0.964 ± 0.153^{a}	36.911 ± 4.176^{c}	36.195 ± 6.968^{c}
Actinobacteridae (subclass)	0.397 ± 0.122^{a}	0.705 ± 0.141^{a}	20.559 ± 2.763^{b}	34.000 ± 7.125^{b}
Coriobacteridae (subclass)	0.169 ± 0.033^{a}	0.258 ± 0.035^{a}	16.276 ± 2.406^{b}	2.190 ± 1.555^{a}
Deferribacteres	0.016 ± 0.005^{a}	0.016 ± 0.007^{a}	0 ± 0^{b}	0 ± 0^{b}
Mollicutes	0.077 ± 0.040^{a}	0.129 ± 0.063^{a}	0 ± 0^{b}	0 ± 0^{b}
Bacilli	0.390 ± 0.138^{a}	0.415 ± 0.113^{a}	1.261 ± 0.471^{b}	3.617 ± 0.897^{c}
unclassified_Bacilli	0.0274 ± 0.0097^{a}	0.0182±0.0070^{a}	0.1951±0.0916^{b}	0.0808±0.0175^{b}
unclassified_Clostridia	0.1000 ± 0.0180^{a}	0.1420±0.0246^{a}	0 ± 0^{b}	0.0010±0.0010^{b}
unclassified_Alphaproteobacteria	0.262 ± 0.085^{a}	0.843 ± 0.252^{a}	0 ± 0^{b}	0 ± 0^{b}
unclassified_Actinobacteria	0 ± 0^{a}	0 ± 0^{a}	0.076 ± 0.023^{b}	0.005 ± 0.003^{a}

Order				
Clostridiales	72.3 ± 1.9[a]	59.8 ± 2.7[b]	28.0 ± 3.5[c]	15.0 ± 4.8[d]
Erysipelotrichales	0.43 ± 0.04[b]	2.08 ± 0.33[a]	3.12 ± 1.56[a,b,c]	9.22 ± 2.32[c]
Lactobacillales	0.36 ± 0.13[a]	0.40 ± 0.11[a,c]	1.07 ± 0.38[c]	3.54 ± 0.88[b]
Verrucomicrobiales	3.677 ± 0.834[b]	8.820 ± 1.621[a]	1.218 ± 1.185[c]	0.009 ± 0.005[c]
Rhodospirillales	0.076 ± 0.011[a]	0.106 ± 0.034[a]	0 ± 0[b]	0 ± 0[b]
Enterobacteriales	0.005 ± 0.002[b]	0.031 ± 0.009[a]	0.145 ± 0.054[a,c]	0.339 ± 0.088[c]
Actinomycetales	0.139 ± 0.028[a]	0.220 ± 0.045[a]	0.058 ± 0.017[b]	0.321 ± 0.119[a]
Micrococcineae (suborder)	0.139 ± 0.028[a]	0.220 ± 0.045[a]	0.058 ± 0.017[b]	0.327 ± 0.117[a]
Bifidobacteriales	0.258 ± 0.123[a]	0.485 ± 0.148[a]	20.495 ± 2.765[b]	33.675 ± 7.012[b]
Coriobacteriales	0.169 ± 0.033[a]	0.258 ± 0.035[a]	16.276 ± 2.406[b]	2.190 ± 1.555[a]
Deferribacterales	0.016 ± 0.005[a]	0.016 ± 0.007[a]	0 ± 0[b]	0 ± 0[b]
Anaeroplasmatales	0.077 ± 0.040[a]	0.129 ± 0.063[a]	0 ± 0[b]	0 ± 0[b]
unclassified_Clostridiales	6.86 ± 2.38[a]	4.66 ± 0.75[a]	0.20 ± 0.06[b]	0.04 ± 0.02[b]
unclassified_Lactobacillales	0.117 ± 0.040[a]	0.172 ± 0.050[a,c]	0.375 ± 0.128[c]	2.200 ± 0.641[b]
unclassified_Rhodospirillales	0.003 ± 0.001[c]	0.010 ± 0.004[a]	0 ± 0[b]	0 ± 0[b]
Family				
Ruminococcaceae	27.7 ± 2.7[a]	22.7 ± 2.3[a]	1.2 ± 0.3[b]	1.2 ± 0.4[b]
Peptostreptococcaceae	6.22 ± 0.99[b]	2.18 ± 1.33[a,c]	3.58 ± 0.74[a,b]	0.35 ± 0.19[c]
Clostridiaceae 1	5.72 ± 1.06[b]	1.37 ± 0.44[a]	2.17 ± 1.17[a]	0.3 ± 0.16[a]
Clostridiales_Incertae Sedis XIII	0.01 ± 0[a]	0.01 ± 0[a]	0 ± 0[b]	0 ± 0[b]
Peptococcaceae 1	0.004 ± 0[a]	0.003 ± 0[a]	0 ± 0[b]	0 ± 0[b]
Erysipelotrichaceae	0.43 ± 0.04[b]	2.08 ± 0.33[a]	3.12 ± 1.56[a,b,c]	9.22 ± 2.32[c]
Lactobacillaceae	0.23 ± 0.09[a,b]	0.13 ± 0.06[a]	0.66 ± 0.26[c]	0.49 ± 0.12[b,c]
Enterococcaceae	0.0031 ± 0.002[c]	0.087 ± 0.0269[a]	0.0284 ± 0.0118[a]	0.8248±0.2128[b]
Porphyromonadaceae	11.364 ± 0.774[a]	8.472 ± 0.925[a]	0.034 ± 0.026[b]	0.007 ± 0.004[b]
Bacteroidaceae	5.44 ± 0.87[b]	10.72 ± 1.78[a]	26.32 ± 4.70[c]	33.17 ± 5.55[c]
Prevotellaceae	2.471 ± 1.286[a]	4.192 ± 1.935[a]	0.002 ± 0.001[b]	0.002 ± 0.002[b]
Rikenellaceae	0.574 ± 0.154[a]	0.974 ± 0.296[a]	0.002 ± 0.002[b]	0.007 ± 0.006[b]

Verrucomicrobiaceae	3.677 ± 0.834[b]	8.820 ± 1.621[a]	1.218 ± 1.185[c]	0.009 ± 0.005[c]
Rhodospirillaceae	0.073 ± 0.022[a]	0.096 ± 0.031[a]	0 ± 0[b]	0 ± 0[b]
Enterobacteriaceae	0.005 ± 0.002[b]	0.031 ± 0.009[a]	0.145 ± 0.054[a,c]	0.339 ± 0.088[c]
Micrococcaceae	0.137 ±0.027[a]	0.220 ± 0.045[a]	0.058 ± 0.017[b]	0.310 ± 0.114[a]
Bifidobacteriaceae	0.258 ± 0.123[a]	0.485 ± 0.148[a]	20.495 ± 2.765[b]	33.675 ± 7.012[b]
Coriobacteriaceae	0.169 ± 0.033[a]	0.258 ± 0.035[a]	16.276 ± 2.406[b]	2.190 ± 1.555[a]
Deferribacteraceae	0.016 ± 0.005[a]	0.016 ± 0.007[a]	0 ± 0[b]	0 ± 0[b]
Anaeroplasmataceae	0.077 ± 0.040[a]	0.129 ± 0.063[a]	0 ± 0[b]	0 ± 0[b]
unclassified_Lachnospiraceae	20.7 ± 2.1[a]	22.6 ± 2.3[a]	4.9 ± 1.9[b]	2.4 ± 0.6[b]
unclassified_Ruminococcaceae	12.13 ± 1.52[a]	11.92 ± 1.73[a]	0.12 ± 0.06[b]	0.1 ± 0.06[b]
unclassified_Clostridiaceae 1	0.08 ± 0.02[a]	0.04 ± 0.01[a,b]	0.06 ± 0.04[b]	0.01 ± 0.01[b]
unclassified_Peptostreptococcaceae	0.15 ± 0.03[b]	0.05 ± 0.04[a]	0.12 ± 0.03[a,b]	0.03 ± 0.02[a]
unclassified_Clostridiales_Incertae Sedis XIII	0.003 ± 0[a]	0.005 ± 0.002[a]	0 ± 0[b]	0 ± 0[b]
unclassified_Erysipelotrichaceae	0.015 ± 0.001[b]	0.106 ± 0.042[a]	0.082 ± 0.064[a,b]	0.173 ±0.048[a]
unclassified_Enterococcaceae	0 ± 0[a]	0.003 ± 0.001[a]	0.002 ± 0.001[a]	0.035 ± 0.008[c]
unclassified_Porphyromonadaceae	4.585 ± 0.620[b]	1.568 ± 0.302[a]	0.005 ± 0.003[c]	0 ± 0[c]
unclassified_Rhodospirillaceae	0.073 ± 0.022[a]	0.095 ± 0.031[a]	0 ± 0[b]	0 ± 0[b]
unclassified_Enterobacteriaceae	0.004 ± 0.002[a]	0.014 ± 0.005[a]	0.061 ± 0.025[a,b]	0.211 ± 0.058[b]
unclassified_Bifidobacteriaceae	0.013 ± 0.006[a]	0.0242 ± 0.011[a]	0.785 ± 0.104[b]	0.494 ± 0.038[c]
unclassified_Coriobacteriaceae	0.131 ± 0.024[b]	0.223 ± 0.031[a]	0.019 ± 0.013[c]	0.007 ± 0.004[c]
Genus				
Dorea	0.13 ±0.09[a]	0.04 ± 0.01[a]	0 ± 0[b]	0 ± 0[b]
Clostridium XlVb	0.38 ± 0.07[a]	0.40 ± 0.06[a]	0 ± 0[b]	0 ± 0[b]
Lachnospiracea_incertae_sedis	0.05 ± 0.01[a]	0.03 ± 0.01[a]	0.24 ± 0.16[a,b]	0 ± 0[b]
Acetatifactor	1.61 ± 0.64[b]	0.25 ± 0.07[a]	0 ± 0[c]	0 ± 0[c]
Roseburia	0.42 ± 0.12[a]	0.51 ± 0.13[a]	0 ± 0[b]	0 ± 0[b]
Coprococcus	0.36 ± 0.21[a]	0.49 ± 0.37[a]	0.01 ± 0.01[b]	0 ± 0[b]
Anaerostipes	0 ± 0[a]	0 ± 0[a]	2.53 ± 1.60[b]	4.76 ± 3.01[b]
Pseudoflavonifractor	0.29 ± 0.04[a]	0.57 ± 0.18[a]	0 ± 0[b]	0 ± 0[b]
Intestinimonas	0.18 ± 0.03[a]	0.22 ± 0.04[a]	0 ± 0[b]	0 ± 0[b]
Acetanaerobacterium	0.12 ± 0.05[c]	0.05 ± 0.05[a]	0.01 ± 0.01[a,b]	0 ± 0[b]

Ruminococcus	12.9 ± 2.4[a]	7.1 ± 1.6[a]	0 ± 0[b]	0.01 ± 0[b]
Clostridium IV	0.50 ± 0.04[a]	0.65 ± 0.06[a]	0.24 ± 0.1[b]	0.19 ± 0.06[b]
Oscillibacter	0.84 ± 0.08[a]	1.28 ± 0.23[a]	0.01 ± 0.01[b]	0.11 ± 0.11[b]
Anaerotruncus	0.09 ± 0.02[a]	0.11 ± 0.03[a]	0 ± 0[b]	0 ± 0[b]
Romboutsia	5.99 ± 0.95[c]	2.08 ± 1.27[a]	3.34 ±0.70[a]	0.30 ± 0.18[b]
Clostridium sensu stricto	5.64 ± 1.05[b]	1.34 ± 0.43[a]	2.11 ± 1.3[a]	0.3 ± 0.15[a]
Eubacterium	0.005 ± 0.003[a]	0.007 ± 0.004[a]	0 ± 0[b]	0 ± 0[b]
Anaerovorax	0.011 ± 0[a]	0.004 ± 0[a]	0 ± 0[b]	0 ± 0[b]
Peptococcus	0.003 ± 0.002[a]	0.003 ± 0.001[a]	0 ± 0[b]	0 ± 0[b]
Clostridium XVIII	0.30 ± 0.05[b]	1.09 ± 0.17[a]	0.17 ± 0.05[b]	1.22 ± 0.71[a,b]
Erysipelotrichaceae_incertae_sedis	0.021 ± 0.05[b]	0.509 ± 0.165[a]	2.817 ± 1.488[a,c]	7.803 ± 2.452[c]
Turicibacter	0.080 ± 0.020[b]	0.003 ± 0.003[a]	0.04 ± 0.015[b]	0 ± 0[a]
Coprobacillus (p value for C vs A is 0.063)	0.011 ± 0.007[a,b]	0.363 ± 0.170[a]	0.004 ± 0.004[b]	0.015 ± 0.015[b]
Lactobacillus	0.23 ± 0.09[a,b]	0.13 ± 0.06[a]	0.66 ± 0.26[c]	0.49 ± 0.12[b,c]
Enterococcus	0.0031 ± 0.002[b]	0.0835±0.0268[a]	0.0260±0.0107[a,b]	0.7869±0.2059[c]
Odoribacter	0.81 ± 0.29[a]	0.92 ± 0.15[a]	0 ± 0[b]	0 ± 0[b]
Parabacteroides	3.885 ± 0.691[a]	3.663 ± 0.694[a]	0.029 ± 0.023[b]	0.006 ± 0.004[b]
Barnesiella	2.08 ± 0.35[a]	2.33 ± 0.61[a]	0 ± 0[b]	0 ± 0[b]
Bacteroides	5.44 ± 0.87[b]	10.72 ± 1.78[a]	26.32 ± 4.70[c]	33.17 ± 5.55[c]
Alloprevotella	2.405 ± 1.295[a]	4.171 ± 1.936[a]	0.002 ± 0.001[b]	0.002 ± 0.002[b]
Alistipes	0.573 ± 0.154[a]	0.974 ± 0.296[a]	0.002 ± 0.002[b]	0.007 ± 0.006[b]
Akkermansia	3.678 ± 0.834[b]	8.820 ± 1.621[a]	1.218 ± 1.185[c]	0.009 ± 0.004[c]
Escherichia/Shigella	0.001 ± 0.001[b]	0.014 ± 0.005[a]	0.077 ± 0.047[a,b,c]	0.117 ± 0.036[c]
Rothia	0.137 ±0.027[a]	0.220 ± 0.045[a]	0.058 ± 0.017[b]	0.310 ± 0.113[a]
Bifidobacterium	0.245 ± 0.123[a]	0.460 ± 0.138[a]	19.710 ± 2.749[b]	33.181 ± 7.034[b]
Parvibacter	0.010 ± 0.004[a]	0.004 ± 0.002[a]	0 ± 0[b]	0 ± 0[b]
Asaccharobacter	0.016 ± 0.005[a]	0.023 ± 0.005[a]	0 ± 0[b]	0.001 ± 0.001[b]
Collinsella	0.009 ± 0.008[a]	0.003 ± 0.002[a]	16.251 ± 2.408[b]	2.167 ± 1.553[c]
Mucispirillum	0.016 ± 0.005[a]	0.016 ± 0.007[a]	0 ± 0[b]	0 ± 0[b]
Anaeroplasma	0.077 ± 0.040[a]	0.129 ± 0.063[a]	0 ± 0[b]	0 ± 0[b]

OTUs				
OTU _61 (bacterium NLAE-zl-H2; JX006253) **99%**	2.031 ± 0.551[a]	1.448 ± 0.471[a]	0.001 ± 0.001[b]	0.083 ± 0.083[b]
OTU _5 (Bacteroides sp. ASF519; ASF 519; AF157056) **100%**	4.931 ± 0.847[a]	4.154 ± 0.754[a]	0.030 ± 0.023[b]	0.006 ± 0.004[b]
OTU _6 (Clostridium disporicum (T); DSM 5521; Y18176) **99%**	7.464 ± 1.374[b]	1.600 ± 0.528[a]	2.090 ± 1.192[a]	0.152 ± 0.067[c]
OTU _52 (bacterium ASF500; ASF 500; AF157051) **91%**	0.590 ± 0.148[a]	0.671 ± 0.155[a]	0 ± 0[b]	0.001 ± 0.001[b]
OTU _120 (rumen bacterium YS2; AF544207) **92%**	0.261 ± 0.072[a]	0.218 ± 0.084[a]	0 ± 0[b]	0.001 ± 0.001[b]
OTU _24 (Gram-negative bacterium cL10-2b-4; AY239469) **89%**	1.893 ± 0.630[a]	1.902 ± 0.798[a]	0 ± 0[b]	0 ± 0[b]
OTU _92 (butyrate-producing bacterium M62/1; AY305309) **97%**	0.140 ± 0.023[c]	0.484 ± 0.133[a]	0 ± 0[b]	0.001 ± 0.001[b]
OTU _62 (Odoribacter laneus; JCM 16069; AB547648) **99%**	0.985 ± 0.347[a]	1.043 ± 0.176[a]	0 ± 0[b]	0 ± 0[b]
OTU_29 (Lachnospiraceae bacterium 19gly4; AF550610) **91%**	7.854 ± 2.153[a]	3.478 ± 1.634[a]	0.006 ± 0.005[b]	0 ± 0[b]
OTU_56 (Insolitispirillum peregrinum subsp. integrum; IAM 14946; AB074521) (p value between C and A is 0.11) **87%**	0.360 ± 0.120[a]	1.014 ± 0.301[a]	0 ± 0[b]	0 ± 0[b]
OTU_30 (Gram-negative bacterium cL10-2b-4; AY239469) **90%**	5.644 ± 0.822[b]	1.629 ± 0.406[a]	0.003 ± 0.002[c]	0 ± 0[c]
OTU_3 (Akkermansia muciniphila (T); Muc; AY271254) (p value between C and A is 0.09) **100%**	4.788 ± 1.104[a]	10.132 ± 1.883[a]	1.255 ± 1.222[b]	0.008 ± 0.005[b]
OTU_12 (Bifidobacterium choerinum; 7 VI A; JQ002525) **99%**	0.325 ± 0.165[a]	0.568 ± 0.183[a]	22.022 ± 3.338[b]	35.979 ± 7.788[b]
OTU_80 (Clostridium sp. Clone-17; AB622837) **98%**	0.146 ± 0.014[a]	0.424 ± 0.199[a]	0 ± 0[b]	0.001 ± 0.001[b]
OTU_35 (Roseburia hominis A2-183; CP003040) **96%**	0.269 ± 0.088[a]	0.105 ± 0.048[a]	0 ± 0[b]	0.001 ± 0.001[b]
OTU_91 (rumen bacterium NK4A214; GU324404) **99%**	0.460 ± 0.141[a]	0.169 ± 0.070[a]	0 ± 0[b]	0 ± 0[b]
OTU_74 (Clostridiales bacterium CIEAF 020; AB702928) **97%**	1.158 ± 0.678[a]	0.022 ± 0.012[a]	0 ± 0[b]	0 ± 0[b]
OTU_96 (Clostridiales bacterium CIEAF 019; AB702936) **92%**	0.183 ± 0.026[a]	0.171 ± 0.052[a]	0 ± 0[b]	0 ± 0[b]
OTU_37 (Eubacterium coprostanoligenes (T); HL; HM037995) **95%**	1.307 ± 0.474[a]	2.070 ± 0.443[a]	0.001 ± 0.001[b]	0.002 ± 0.002[b]
OTU_44 (Terrisporobacter glycolicus; 2002-87768; AY244773) **100%**	2.414 ± 0.414[b]	0.752 ± 0.555[a]	1.194 ± 0.231[c]	0.131 ± 0.060[a]
OTU_43 (Rothia sp. EGY-WPhB4; KJ545601) **98%**	0.174 ± 0.035[a]	0.250 ± 0.049[a]	0.063 ± 0.018[b]	0.331 ± 0.124[a]
OTU_1 (Bacteroides vulgatus; BCRC12903; EU136687) **100%**	4.096 ± 0.960[c]	9.071 ± 1.682[a]	26.818 ± 4.964[b]	31.952 ± 5.475[b]
OTU_81 (Lachnospiraceae bacterium 605; AB700364) **99%**	0.416 ± 0.131[a]	0.355 ± 0.144[a]	0.006 ± 0.004[b]	0.009 ± 0.008[b]
OTU_291 (Catabacter hongkongensis (T); HKU16; AY574991) **92%**	0.013 ± 0.004[a]	0.004 ± 0.002[a]	0 ± 0[b]	0 ± 0[b]
OTU_723 (Intestinimonas sp. GD4; LN870298) **95%**	0.183 ± 0.041[a]	0.169 ± 0.032[a]	0 ± 0[b]	0 ± 0[b]
OTU_98 (Clostridiales bacterium CIEAF 020; AB702928) **82%**	0.424 ± 0.239[a]	0.122 ± 0.091[a]	0 ± 0[b]	0 ± 0[b]
OTU_106 (Anaeroplasma bactoclasticum (T); JR; ATCC 27112; M25049) **91%**	0.065 ± 0.046[a]	0.112 ± 0.070[a]	0 ± 0[b]	0 ± 0[b]
OTU_4 (Lachnospiraceae bacterium 607; AB700365) **96%**	1.189 ± 0.500[a]	0.629 ± 0.247[a]	0.003 ± 0.001[b]	0.006 ± 0.003[b]

OTU_905 (Clostridium methylpentosum (T); DSM 5476; Y18181) **91%**	0.035 ± 0.011[a]	0.037 ± 0.012[a]	0 ± 0[b]	0 ± 0[b]
OTU_25 (Clostridium sp. Culture-1; AB622814) **99%**	1.305 ± 0.131[a]	1.559 ± 0.362[a]	0 ± 0[b]	0 ± 0[b]
OTU_160 (bacterium YE57; AY442821) **97%**	0.094 ± 0.019[a]	0.089 ± 0.022[a]	0 ± 0[b]	0 ± 0[b]
OTU_254 (Clostridium sp. Clone-7; AB622834) **94%**	0.010 ± 0.004[a]	0.005 ± 0.003[a]	0 ± 0[b]	0 ± 0[b]
OTU_980 (Murimonas intestini (T); SRB530; KC311366) **97%**	0.091 ± 0.025[a]	0.085 ± 0.027[a]	0 ± 0[b]	0 ± 0[b]
OTU_47 (Coprococcus catus; L8; AB361624) **96%**	0.471 ± 0.105[a]	0.494 ± 0.182[a]	0.001 ± 0.001[b]	0 ± 0[b]
OTU_83 (Clostridium sp. Clone-7; AB622834) **93%**	0.197 ± 0.080[a]	0.119 ± 0.055[a]	0 ± 0[b]	0 ± 0[b]
OTU_153 (Clostridium sp. Clone-7; AB622834) **97%**	0.107 ± 0.040[a]	0.032 ± 0.017[a]	0 ± 0[b]	0 ± 0[b]
OTU_253 (Turicibacter sp. LA61; AB727348) **100%**	0.034 ± 0.010[b]	0.002 ± 0.002[a,c]	0.014 ± 0.005[c,b]	0 ± 0[a]
OTU_1240 (Clostridiales bacterium CIEAF 026; AB702939) **97%**	0.155 ± 0.022[a]	0.142 ± 0.045[a]	0 ± 0[b]	0 ± 0[b]
OTU_89 (Clostridium sp. Culture Jar-13; AB622826) **97%**	0.229 ± 0.070[a]	0.099 ± 0.035[a]	0.002 ± 0.001[b]	0 ± 0[b]
OTU_70 (Clostridium cocleatum; CM972; AF028350) **100%**	0.339 ± 0.071[a]	0.825 ± 0.262[a]	0.047 ± 0.047[b]	0 ± 0[b]
OTU_64 (Lactobacillales bacterium HY-36-1; AY581272) **96%**	0.247 ± 0.054[a]	0.284 ± 0.067[a]	0.001 ± 0.001[b]	0 ± 0[b]
OTU_60 (Roseburia sp. 499; JX629259) **97%**	0.727 ± 0.203[a]	0.540 ± 0.180[a]	0.004 ± 0.004[b]	0 ± 0[b]
OTU_59 (Clostridium viride (T); T2-7 (DSM 6836); X81125) **97%**	0.246 ± 0.077[a]	0.304 ± 0.073[a]	0 ± 0[b]	0 ± 0[b]
OTU_227 (Defluviitalea saccharophila (T); LIND6LT2; HQ020487) **92%**	0.056 ± 0.024[a]	0.057 ± 0.017[a]	0 ± 0[b]	0 ± 0[b]
OTU_1195 (Clostridium viride (T); T2-7 (DSM 6836); X81125) **95%**	0.078 ± 0.012[a]	0.131 ± 0.036[a]	0 ± 0[b]	0 ± 0[b]
OTU_207 (Natranaerovirga pectinivora (T); AP3; GQ922846) **92%**	0.028 ± 0.006[c]	0.009 ± 0.005[a]	0 ± 0[b]	0 ± 0[b]
OTU_41 (Ruminococcus sp. DJF_VR67; EU728791) **97%**	3.801 ± 0.573[a]	2.255 ± 0.537[a]	0.016 ± 0.013[b]	0.009 ± 0.008[b]
OTU_22 (Clostridium sp. A9; DQ789119) **98%**	1.086 ± 0.209[a]	0.841 ± 0.194[a]	0.077 ± 0.049[b]	0.001 ± 0.001[b]
OTU_369 (Lachnospiraceae bacterium A4; DQ789118) **96%**	0.818 ± 0.413[a]	0.208 ± 0.138[a]	0 ± 0[b]	0 ± 0[b]
OTU_166 (Lachnospiraceae bacterium 19gly4; AF550610) **94%**	0.034 ± 0.014[a]	0.025 ± 0.009[a]	0 ± 0[b]	0 ± 0[b]
OTU_181 (Clostridium sp. Culture-41; AB622820) **94%**	0.089 ± 0.023[a]	0.048 ± 0.012[a]	0 ± 0[b]	0 ± 0[b]
OTU_99 (Dehalobacterium formicoaceticum; DMC; X86690) **89%**	0.182 ± 0.024[a]	0.186 ± 0.031[a]	0 ± 0[b]	0 ± 0[b]
OTU_128 (Clostridium sp. Culture-54; AB622823) **98%**	0.116 ± 0.018[a]	0.087 ± 0.031[a]	0 ± 0[b]	0 ± 0[b]
OTU_39 (Clostridiales bacterium CIEAF 021; AB702937) **93%**	0.492 ± 0.060[a]	0.641 ± 0.144[a]	0 ± 0[b]	0.001 ± 0.001[b]
OTU_220 (Ruminococcaceae bacterium mt9; LN866991) **95%**	0.102 ± 0.021[a]	0.057 ± 0.018[a]	0.001 ± 0.001[b]	0.001 ± 0.001[b]
OTU_45 (Clostridium sp. Culture Jar-8; AB622825) **98%**	0.075 ± 0.016[a]	0.038 ± 0.013[a]	0 ± 0[b]	0 ± 0[b]
OTU_188 (Coriobacterium glomerans (T); DSM 20642; X79048) **89%** (p value between C and A is 0.06)	0.031 ± 0.007[a]	0.053 ± 0.008[a]	0.001 ± 0.001[b]	0.001 ± 0.001[b]
OTU_26 (Oscillibacter sp. G2; HM626173) **95%**	0.475 ±0.095[a]	0.513 ± 0.151[a]	0.001 ± 0.001[b]	0.001 ± 0.001[b]
OTU_212 (Clostridiales bacterium CIEAF 019; AB702936) **94%**	0.018 ± 0.005[a]	0.009 ± 0.003[a]	0 ± 0[b]	0 ± 0[b]

OTU_215 (Clostridiales bacterium CIEAF 021; AB702937) **97%**	0.067 ± 0.024^{a}	0.138 ± 0.059^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_113 (Clostridium sp. ID5; AY960574) **98%**	0.325 ± 0.053^{a}	0.204 ± 0.016^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_140 (Peptostreptococcaceae bacterium canine oral taxon 333; 1G023; JN713503) **95%**	0.101 ± 0.019^{a}	0.146 ± 0.018^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_114 (Clostridium sp. Clone-49; AB622849) **99%** (p value between C and A is 0.14)	0.0141 ± 0.028^{a}	0.104 ± 0.044^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_84 (Clostridium sp. BPY5; KM360180) **97%**	0.231 ± 0.041^{a}	0.123 ± 0.046^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_204 (Lachnospiraceae bacterium AIP 541.12; KF814113) **94%**	0.139 ± 0.033^{a}	0.062 ± 0.033^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_54 (Lachnospiraceae bacterium 610; AB700367) **99%**	0.306 ± 0.215^{a}	0.440 ± 0.265^{a}	0.001 ± 0.001^{b}	0 ± 0^{b}
OTU_49 (Alistipes finegoldii; CIP 107999; AY643084) **100%**	0.740 ± 0.208^{a}	1.114 ± 0.325^{a}	0.002 ± 0.002^{b}	0.008 ± 0.006^{b}
OTU_237 (rumen bacterium R-7; AB239481) **94%**	0.031 ± 0.008^{a}	0.018 ± 0.006^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_378 (bacterium ASF500; ASF 500; AF157051) **97%**	0.360 ± 0.052^{a}	0.587 ± 0.197^{a}	0.001 ± 0.001^{b}	0 ± 0^{b}
OTU_708 (Intestinimonas butyriciproducens AP4; JX101685) **97%** (p value between C and A is 0.14)	0.275 ± 0.053^{a}	0.488 ± 0.131^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_85 (Roseburia intestinalis (T); L1-82; AJ312385) **99%**	0.518 ± 0.137^{a}	0.595 ± 0.156^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_132 (Oscillospira guilliermondii; OSC3; AB040497) **96%**	0.071 ± 0.017^{a}	0.081 ± 0.019^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_195 (Candidatus Soleaferrea massiliensis AP7; JX101688) **93%**	0.010 ± 0.002^{a}	0.009 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_642 (Clostridiales bacterium 24-4c; HQ452852) **95%**	0.052 ± 0.016^{a}	0.110 ± 0.045^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_248 (Gordonibacter pamelaeae 7-10-1-b; 7-10-1-bT; FP929047) **94%**	0.010 ± 0.004^{a}	0.013 ± 0.003^{a}	0.001 ± 0.001^{b}	0.001 ± 0.001^{b}
OTU_182 (Lachnospiraceae bacterium TWA4; TWA4_69_rrs; JN196964) **95%**	0.057 ± 0.010^{a}	0.055 ± 0.014^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_167 (Barnesiella viscericola DSM 18177; C46, DSM 18177; CP007034) **91%**	0.212 ± 0.035^{a}	0.140 ± 0.021^{a}	0.001 ± 0.001^{b}	0 ± 0^{b}
OTU_250 (butyrate-producing bacterium SS3/4; AY305316) **97%**	0.015 ± 0.005^{a}	0.007 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_465 (Clostridium sp. YIT 12070; AB491208) **94%**	0.037 ± 0.012^{a}	0.110 ± 0.031^{c}	0 ± 0^{b}	0 ± 0^{b}
OTU_485 (Candidatus Soleaferrea massiliensis AP7; JX101688) **93%**	$0.002 \pm 0.002^{a,b}$	0.006 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_386 (Anaerotruncus sp. MT15; LN881593) **93%**	0.022 ± 0.006^{a}	0.007 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_50 (Intestinimonas sp. FSAA-17; KP114242) **96%**	0.305 ± 0.070^{a}	0.262 ± 0.073^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_79 (Clostridiales bacterium CIEAF 015; AB702929) **93%**	0.040 ± 0.011^{a}	0.014 ± 0.007^{c}	0 ± 0^{b}	0 ± 0^{b}
OTU_108 (Clostridium sp. Culture-41; AB622820) **94%**	0.084 ± 0.020^{a}	0.120 ± 0.035^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_110 (bacterium ASF500; ASF 500; AF157051) **94%**	0.131 ± 0.021^{a}	0.225 ± 0.044^{a}	0.001 ± 0.001^{b}	0 ± 0^{b}

OTU_152 (Adlercreutzia equolifaciens; FJC-B20; AB306662) **99%** (p value between C and A is 0.06)	0.109 ± 0.022^{a}	0.185 ± 0.028^{a}	0.017 ± 0.012^{b}	0.005 ± 0.005^{b}
OTU_375 (Clostridiales bacterium CIEAF 021; AB702937) **100%**	0.051 ± 0.011^{a}	0.033 ± 0.011^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_111 (Clostridium sp. AT5; LN881614) **97%**	0.084 ± 0.019^{a}	0.148 ± 0.40^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_90 (Hydrogenoanaerobacterium saccharovorans (T); SW512; EU158190) **93%**	0.044 ± 0.019^{a}	0.024 ± 0.010^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_199 (butyrate-producing bacterium SM4/1; AY305314) **97%**	0.060 ± 0.026^{a}	0.210 ± 0.116^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_419 (Clostridium sp. BPY5; KM360180) **95%**	0.011 ± 0.005^{b}	$0.002 \pm 0.001^{a,b}$	0 ± 0^{a}	0 ± 0^{a}
OTU_265 (Clostridiales bacterium CIEAF 015; AB702929) **93%**	0.049 ± 0.025^{a}	0.061 ± 0.033^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_142 (Aestuariispira insulae (T); AH-MY2; KF876014) **86%**	0.100 ± 0.016^{a}	0.103 ± 0.038^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_130 (Clostridium sp. Culture-41; AB622820) **96%**	0.076 ± 0.019^{a}	0.134 ± 0.065^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_527 (Intestinimonas butyriciproducens AP4; JX101685) **96%** (p value between C and A is 0.06)	0.038 ± 0.006^{a}	0.087 ± 0.018^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_421 (Clostridium sp. ASF356; ASF 356; AF157052) **94%**	0.010 ± 0.004^{b}	$0.002 \pm 0.001^{a,b}$	0 ± 0^{a}	0 ± 0^{a}
OTU_125 (Clostridium sp. Culture-41; AB622820) **98%**	0.063 ± 0.030^{a}	0.092 ± 0.038^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_126 (Caminicella sporogenes (T); AM1114; AJ320233) **88%**	0.031 ± 0.008^{a}	0.042 ± 0.010^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_155 (Phycicoccus sp. N5d-3; GQ344407) **85%**	0.023 ± 0.009^{a}	0.017 ± 0.008^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_300 (rumen bacterium R-7; AB239481) **91%**	0.020 ± 0.006^{a}	0.013 ± 0.005^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_145 (Clostridiales bacterium 21-4c; HQ452858) **95%**	0.364 ± 0.121^{a}	0.458 ± 0.079^{a}	0.001 ± 0.001^{b}	0 ± 0^{b}
OTU_159 (Clostridium viride (T); T2-7 (DSM 6836); X81125) **97%**	0.055 ± 0.008^{a}	0.083 ± 0.020^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_174 (Intestinimonas butyriciproducens AP4; JX101685) **97%**	0.106 ± 0.037^{a}	0.064 ± 0.016^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_214 (Lactobacillus murinus; ONS2; AY324630) **100%**	$0.116 \pm 0.074^{a,b}$	0.028 ± 0.014^{a}	0.281 ± 0.109^{b}	0.061 ± 0.031^{a}
OTU_147 (Clostridium methylpentosum (T); DSM 5476; Y18181) **91%**	0.038 ± 0.006^{a}	0.143 ± 0.059^{a}	0.006 ± 0.006^{b}	0 ± 0^{b}
OTU_194 (Murimonas intestini (T); SRB530; KC311366) **98%**	0.522 ± 0.234^{a}	0.047 ± 0.012^{a}	0.001 ± 0.001^{b}	0.001 ± 0.001^{b}
OTU_136 (Anaerotruncus colihominis (T); 14565; AJ315980) **96%**	0.063 ± 0.029^{a}	0.073 ± 0.016^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_34 (Ruminococcus flavefaciens; C1a; AM915271) **98%**	0.046 ± 0.018^{a}	0.013 ± 0.006^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_902 (Clostridiales bacterium oral taxon F32; VO026; HM099644) **96%**	0.013 ± 0.003^{a}	0.030 ± 0.013^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_133 (Kocuria sp. SA14; KJ599867) **81%**	0.030 ± 0.010^{a}	0.016 ± 0.007^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_148 (Clostridium sp. ID6; AY960572) **97%**	0.032 ± 0.017^{a}	0.010 ± 0.004^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_138 (Intestinimonas sp. GD2; LN866996) **95%**	0.035 ± 0.010^{a}	0.025 ± 0.009^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_692 (Syntrophococcus sp. BS-2; GU045475) **94%**	0.205 ± 0.107^{a}	0.139 ± 0.058^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_274 (Peptostreptococcaceae bacterium canine oral taxon 303;	0.030 ± 0.013^{a}	0.043 ± 0.014^{a}	0 ± 0^{b}	0 ± 0^{b}

ZY090; JN713469) **94%**				
OTU_280 (Peptostreptococcaceae bacterium oral taxon 091; _X028; GU400649) **92%**	0.010 ± 0.004^{a}	0.008 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_168 (Clostridium sp. Clone-7; AB622834) **97%**	0.032 ± 0.006^{a}	0.031 ± 0.011^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_180 (Clostridium sp. ASF502; ASF 502; AF157053) **98%**	0.049 ± 0.033^{a}	0.088 ± 0.068^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_219 (Clostridiales bacterium CIEAF 016; AB702930) **99%**	0.021 ± 0.003^{a}	0.061 ± 0.020^{c}	0.006 ± 0.005^{b}	$0.024 \pm 0.016^{a,b}$
OTU_100 (butyrate-producing bacterium M62/1; AY305309) **97%**	0.070 ± 0.023^{a}	1.209 ± 0.758^{a}	0.001 ± 0.001^{b}	0 ± 0^{b}
OTU_571 (butyrate-producing bacterium SM4/1; AY305314) **97%**	0.041 ± 0.13^{a}	0.027 ± 0.018^{c}	0 ± 0^{b}	0 ± 0^{b}
OTU_10 (Lachnospiraceae bacterium G11; KC143064) **99%**	0.439 ± 0.275^{a}	0.441 ± 0.317^{a}	0 ± 0^{b}	0.002 ± 0.001^{b}
OTU_36 (Clostridium aff. innocuum CM970; AF028352) **100%**	0.019 ± 0.006^{b}	0.541 ± 0.203^{a}	$3.104 \pm 1.649^{a,c}$	8.393 ± 2.618^{c}
OTU_51 (Clostridiales bacterium oral taxon F32; VO026; HM099644) **93%**	3.685 ± 2.925^{a}	0.684 ± 0.433^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_175 (bacterium ASF500; ASF 500; AF157051) **92%**	0.064 ± 0.013^{a}	0.053 ± 0.011^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_223 (Clostridiales bacterium oral taxon F32; VO026; HM099644) **92%**	0.061 ± 0.032^{a}	0.027 ± 0.012^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_252 (Clostridiales bacterium P16; AB730732) **94%**	0.151 ± 0.058^{a}	0.038 ± 0.007^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_8 (Lachnospiraceae bacterium 609; AB700366) **97%**	2.783 ± 1.097^{a}	6.795 ± 1.448^{c}	0.002 ± 0.001^{b}	0.002 ± 0.002^{b}
OTU_418 (Clostridium sp.; LIP5; Y12289) **96%**	0.050 ± 0.025^{b}	$0.004 \pm 0.003^{a,b}$	0 ± 0^{a}	0 ± 0^{a}
OTU_78 (Clostridiales bacterium CIEAF 017; AB702931) **99%**	0.045 ± 0.019^{a}	2.187 ± 0.714^{c}	0 ± 0^{b}	0 ± 0^{b}
OTU_150 (Clostridiales bacterium CIEAF 015; AB702929) **93%**	0.075 ± 0.032^{a}	0.261 ± 0.193^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_68 (Ruminococcus faecis (T); Eg2; FJ611794) **97%**	0.059 ± 0.024^{a}	$0.022 \pm 0.013^{a,b}$	0.015 ± 0.015^{b}	0.014 ± 0.014^{b}
OTU_263 (butyrate-producing bacterium L2-12; AJ270488) **98%**	0.021 ± 0.008^{a}	0.018 ± 0.006^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_157 (Lachnospiraceae bacterium 19gly4; AF550610) **99%**	0.025 ± 0.004^{a}	0.022 ± 0.005^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_367 (Parvibacter caecicola (T); NR06; GQ456228) **99%**	0.014 ± 0.006^{a}	0.005 ± 0.002^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_314 (Flexistipes group bacterium HRI1cae; AF059187) **100%**	0.005 ± 0.002^{a}	0.008 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_31 (Clostridium sp. YIT 12069; AB491207) **88%**	0.890 ± 0.344^{a}	1.986 ± 0.813^{a}	0.001 ± 0.001^{b}	0 ± 0^{b}
OTU_523 (Clostridiales bacterium CIEAF 013; AB702935) **97%**	0.017 ± 0.004^{a}	0.010 ± 0.004^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_189 (Anaerotruncus colihominis (T); 14565; AJ315980) **100%**	0.017 ± 0.007^{a}	0.033 ± 0.016^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_1140 (Clostridiales bacterium CIEAF 026; AB702939) **96%**	0.007 ± 0.002^{a}	0.007 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_2 (Alloprevotella rava; F0323; GU470887) **87%**	2.990 ± 1.631^{a}	4.892 ± 2.247^{a}	0.002 ± 0.001^{b}	0.002 ± 0.002^{b}
OTU_711 (Clostridium sp. Clone-7; AB622834) **96%**	0.014 ± 0.003^{a}	0.022 ± 0.012^{a}	0 ± 0^{b}	0.001 ± 0.001^{b}
OTU_200 (Clostridiales bacterium CIEAF 026; AB702939) **89%**	0.012 ± 0.004^{b}	$0.013 \pm 0.009^{a,b}$	0 ± 0^{a}	0 ± 0^{a}
OTU_996 (Clostridium sphenoides; DSM 632; X73449) **94%**	0.006 ± 0.002^{a}	0.015 ± 0.010^{a}	0 ± 0^{b}	0 ± 0^{b}

OTU_121 (Clostridium ramosum; CM-C50; EU869233) **100%**	0.003 ± 0.001^{a}	0.006 ± 0.003^{a}	0.121 ± 0.033^{b}	0.969 ± 0.660^{b}
OTU_309 (Candidatus Soleaferrea massiliensis AP7; JX101688) **98%**	0.015 ± 0.003^{a}	0.019 ± 0.011^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_323 (Dehalobacterium formicoaceticum; DMC; X86690) **88%**	$0.002 \pm 0.001^{a,b}$	0.003 ± 0.001^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_304 (Blautia sp. canine oral taxon 143; PP006; JN713310) **97%**	0.039 ± 0.013^{a}	0.037 ± 0.017^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_245 (Adlercreutzia equolifaciens; FJC-M48; AB434709) **95%**	0.013 ± 0.003^{a}	0.014 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_184 (Clostridium leptum (T); DSM 753T; AJ305238) **97%**	0.076 ± 0.028^{a}	0.162 ± 0.034^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_506 (Clostridium sp. cTPY-17; AY239462) **99%**	0.223 ± 0.143^{a}	0.358 ± 0.111^{a}	0.001 ± 0.001^{b}	0.028 ± 0.028^{b}
OTU_176 (Clostridium sp. Culture-41; AB622820) **95%** (p value between C and A is 0.09)	0.092 ± 0.047^{a}	0.290 ± 0.091^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_170 (Clostridiaceae bacterium NML 061030; EU183300) **100%**	$0.048 \pm 0.018^{a,b}$	0.152 ± 0.074^{a}	$0.045 \pm 0.027^{a,b}$	0.044 ± 0.039^{b}
OTU_1147 (Clostridium sp. Clone-49; AB622849) **97%**	0.014 ± 0.005^{a}	$0.049 \pm 0.045^{a,b}$	0 ± 0^{b}	0 ± 0^{b}
OTU_310 (Clostridium sp. SL-2013-71; HG326494) **93%**	0.006 ± 0.003^{a}	0.008 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_190 (Candidatus Soleaferrea massiliensis AP7; JX101688) **93%**	0.020 ± 0.009^{a}	0.051 ± 0.016^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_119 (Flavonifractor plautii; DSM 6740; Y18187) **100%**	0.005 ± 0.003^{a}	0.067 ± 0.027^{c}	0.915 ± 0.213^{b}	0.767 ± 0.323^{b}
OTU_123 (Lachnospiraceae bacterium A2; DQ789117) **97%**	0.015 ± 0.008^{a}	0.042 ± 0.022^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_330 (Escherichia coli; PK3; X80731) **100%**	0.001 ± 0.001^{b}	0.017 ± 0.006^{a}	$0.088 \pm 0.056^{a,b,c}$	0.125 ± 0.037^{s}
OTU_318 (Candidatus Soleaferrea massiliensis AP7; JX101688) **96%**	0.017 ± 0.008^{b}	0.054 ± 0.014^{a}	0.027 ± 0.027^{b}	0.012 ± 0.012^{b}
OTU_122 (Barnesiella intestinihominis (T); YIT 11860; AB370251) **86%**	0.687 ± 0.309^{a}	0.834 ± 0.385^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_862 (Clostridiaceae bacterium DJF_LS40; EU728744) **96%**	$0.007 \pm 0.005^{a,b}$	0.009 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_383 (Intestinimonas sp. GD4; LN870298) **95%**	0.003 ± 0.002^{a}	0.006 ± 0.002^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_972 (Clostridium sp. AT5; LN881614) **98%**	0.005 ± 0.002^{a}	0.013 ± 0.008^{a}	0 ± 0^{b}	$0.001 \pm 0.001^{a,b}$
OTU_233 (Enterococcus durans (T); DSM20633; AJ276354) **100%**	0.004 ± 0.003^{b}	0.105 ± 0.032^{a}	$0.030 \pm 0.012^{a,b}$	0.881 ± 0.231^{c}
OTU_1056 (butyrate-producing bacterium SM4/1; AY305314) **97%**	0.006 ± 0.004^{b}	0.031 ± 0.011^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_297 (bacterium NLAE-zl-G513; JX048499) **95%**	0.006 ± 0.001^{a}	0.009 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_396 (Ruminococcus bromii; X85099) **89%**	$0.006 \pm 0.003^{a,b}$	0.009 ± 0.004^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_238 (rumen bacterium YS2; AF544207) **94%**	0.013 ± 0.003^{a}	0.040 ± 0.015^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_380 (Christensenella minuta (T); YIT 12065; AB490809) **83%**	$0.002 \pm 0.001^{a,b}$	0.007 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}

OTU_1003 (Clostridium sp. BPY5; KM360180) **97%**	0.025 ± 0.014^{a}	0.068 ± 0.023^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_415 (bacterium NLAE-zl-H61; JX006307) **99%**	0.012 ± 0.004^{b}	0.070 ± 0.019^{a}	0.037 ± 0.037^{b}	0.074 ± 0.073^{b}
OTU_251 (Oscillibacter valericigenes (T); Sjm18-20 (= NBRC 101213); AB238598) **96%** (p value between C and A is 0.06)	0.040 ± 0.019^{a}	0.154 ± 0.053^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_256 (Candidatus Stoquefichus sp. SB1; LN850736) **95%**	0.013 ± 0.011^{b}	0.098 ± 0.053^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_379 (Anaerotruncus colihominis (T); 14565; AJ315980) **94%**	0.008 ± 0.004^{a}	0.006 ± 0.002^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_349 (Clostridiales bacterium GluBS11; KP233894) **95%**	0.015 ± 0.008^{a}	0.010 ± 0.004^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_655 (Clostridium sp. AT5; LN881614) **94%**	0.001 ± 0.001^{b}	0.008 ± 0.002^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_413 (Clostridium sp. JC3; AB093546) **90%** (p value between C and A = 0.08)	$0.002 \pm 0.001^{a,b}$	0.008 ± 0.002^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_290 (Clostridiales bacterium CIEAF 013; AB702935) **95%**	0 ± 0^{b}	0.007 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_293 (Candidatus Soleaferrea massiliensis AP7; JX101688) **95%**	0.005 ± 0.002^{a}	0.010 ± 0.004^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_316 (Lachnospiraceae bacterium 19gly4; AF550610) **94%**	0.028 ± 0.016^{a}	0.036 ± 0.016^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_261 (Eubacterium sp. WAL 14571; FJ687606) **94%** (p value between C and A = 0.06)	0.017 ± 0.007^{a}	0.033 ± 0.007^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_282 (Clostridiaceae bacterium FH052; AB298768) **95%**	0.008 ± 0.003^{a}	0.007 ± 0.002^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_544 (Christensenella minuta (T); YIT 12065; AB490809) **88%**	0 ± 0^{b}	0.004 ± 0.002^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_442 (Anaerotruncus colihominis; HKU19; DQ002932) **94%**	$0.005 \pm 0.003^{a,b}$	0.009 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_410 (Dielma fastidiosa; JC13; JF824807) **92%**	$0.001 \pm 0.001^{a,b}$	0.004 ± 0.001^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_277 (Peptostreptococcaceae bacterium canine oral taxon 074; OC009; JN713238) **93%**	0.013 ± 0.004^{a}	0.015 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_1218 (Blautia glucerasea (T); HFTH-1; AB439724) **97%**	0.001 ± 0.001^{b}	0.012 ± 0.004^{a}	0 ± 0^{b}	0.008 ± 0.008^{b}
OTU_394 (Clostridium sp. Culture Jar-13; AB622826) **95%**	0.018 ± 0.013^{a}	0.014 ± 0.007^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_192 (Clostridium sp.; DR6A; Y10028) **100%**	0 ± 0^{b}	0.007 ± 0.003^{a}	0.090 ± 0.044^{a}	0.231 ± 0.177^{a}
OTU_463 (Lachnospiraceae bacterium DJF_RP14; EU728751) **97%**	0.048 ± 0.026^{a}	0.018 ± 0.010^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_342 (Peptostreptococcaceae bacterium canine oral taxon 221; PV088; JN713384) **95%**	0.015 ± 0.004^{a}	0.014 ± 0.004^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_399 (Ruminococcaceae bacterium HZ254R; JN656278) **89%**	0.012 ± 0.006^{a}	0.032 ± 0.021^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_71 (Clostridiales bacterium oral taxon F32; VO026; HM099644) **95%**	0.428 ± 0.166^{a}	0.756 ± 0.220^{a}	0 ± 0^{b}	0.001 ± 0.001^{b}
OTU_179 (Eubacterium dolichum; JCM 10413; AB649277) **92%**	0.010 ± 0.004^{a}	0.059 ± 0.027^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_134 (Clostridiales bacterium JN18_V41_S; DQ168656) **95%**	0.029 ± 0.010^{a}	0.021 ± 0.008^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_104 (Roseburia sp. 499; JX629259) **96%**	0.051 ± 0.021^{b}	$0.029 \pm 0.018^{a,b}$	0 ± 0^{a}	0 ± 0^{a}

OTU_583 (Ruminococcus bromii; X85099) **99%**	0.774 ± 0.718^{a}	0.157 ± 0.043^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_203 (Intestinimonas sp. GD4; LN870298) **94%**	$0.004 \pm 0.004^{a,b}$	0.019 ± 0.009^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_232 (Clostridiales bacterium canine oral taxon 219; PV085; JN713382) **91%**	0.020 ± 0.009^{a}	0.030 ± 0.012^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_373 (Natranaerovirga pectinivora (T); AP3; GQ922846) **90%**	0 ± 0^{b}	0.008 ± 0.004^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_224 (Clostridiales bacterium P16; AB730732) **95%**	0.014 ± 0.005^{a}	0.013 ± 0.008^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_217 (Anaerotruncus sp. MT15; LN881593) **93%**	0.013 ± 0.005^{a}	0.029 ± 0.011^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_271 (butyrate-producing bacterium SL7/1; AY305312) **98%**	0.036 ± 0.020^{a}	0.089 ± 0.043^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_23 (Coprococcus eutactus (T); ATCC 27759; EF031543) **97%**	2.075 ± 1.486^{a}	0.909 ± 0.492^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_359 (Clostridium sp. YIT 12069; AB491207) **98%**	0.006 ± 0.003^{a}	0.010 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_101 (Desulfonosporus sp. AAN04; AB436739) **83%**	0.034 ± 0.012^{c}	0.085 ± 0.017^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_20 (Clostridium sp. Clone-40; AB622844) **97%**	0.258 ± 0.108^{c}	0.023 ± 0.011^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_595 (Lachnospiraceae bacterium 14-2; DQ789124) **98%**	0.021 ± 0.011^{b}	$0.037 \pm 0.026^{a,b}$	0 ± 0^{a}	0 ± 0^{a}
OTU_206 (bacterium ic1340; DQ057467) **95%**	0.028 ± 0.006^{a}	0.172 ± 0.071^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_241 (Ruminococcaceae bacterium GD6; LN881596) **92%**	0.009 ± 0.005^{c}	0.016 ± 0.004^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_366 (Roseburia intestinalis; XB6B4; AM055815) **95%**	0.004 ± 0.002^{a}	0.004 ± 0.002^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_286 (Denitrobacterium detoxificans; NPOH3; AF079506) **89%**	0.005 ± 0.003^{a}	0.004 ± 0.002^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_177 (Clostridiales bacterium CIEAF 021; AB702937) **94%**	0.015 ± 0.006^{a}	0.011 ± 0.006^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_186 (Anaerotruncus colihominis; HKU19; DQ002932) **94%**	0.022 ± 0.008^{a}	0.008 ± 0.004^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_354 ([Clostridium] aldenense; W16015C2; KP944172) **95%**	0.011 ± 0.004^{a}	0.006 ± 0.002^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_372 (Clostridium sp. YIT 12070; AB491208) **94%**	0 ± 0^{b}	0.032 ± 0.015^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_989 (Clostridium sp. Culture-41; AB622820) **96%**	0.008 ± 0.003^{b}	$0.004 \pm 0.003^{a,b}$	0 ± 0^{a}	0 ± 0^{a}
OTU_333 (Defluviitalea saccharophila (T); LIND6LT2; HQ020487) **88%**	$0.003 \pm 0.002^{a,b}$	0.011 ± 0.005^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_341 (bacterium NLAE-zl-C231; JQ608310) **93%**	0.004 ± 0.002^{a}	0.005 ± 0.002^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_540 (Anaerosporobacter mobilis (T); IMSNU 40011; AY534872) **95%**	0 ± 0^{b}	0.010 ± 0.004^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_165 (Clostridium papyrosolvens (T); DSM 2782; X71852) **92%**	$0.005 \pm 0.003^{a,b}$	0.006 ± 0.002^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_439 (Acetivibrio cellulolyticus; L35515) **92%**	0.008 ± 0.003^{b}	$0.001 \pm 0.001^{a,b}$	0 ± 0^{a}	0 ± 0^{a}
OTU_497 (Clostridium paraputrificum; YE51; AY442815) **99%**	0.001 ± 0.001^{a}	0.001 ± 0.001^{a}	0.073 ± 0.030^{b}	0.029 ± 0.009^{b}
OTU_298 (Clostridiales bacterium CIEAF 022; AB702938) **89%**	0.018 ± 0.004^{a}	0.016 ± 0.009^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_510 (Ruminococcaceae bacterium mt9; LN866991) **94%**	0 ± 0^{b}	0.003 ± 0.001^{a}	0 ± 0^{b}	0 ± 0^{b}

OTU_18 (Collinsella aerofaciens; G118; AJ245919) **100%**	0.010 ± 0.008^{a}	0.004 ± 0.002^{a}	17.061 ± 2.749^{b}	2.184 ± 1.567^{c}
OTU_454 (Clostridiales bacterium 80/3; EU266551) **98%**	0.026 ± 0.018^{a}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}
OTU_303 (Clostridiaceae bacterium FH042; AB298771) **96%**	0.006 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}
OTU_88 (Clostridium indolis; CM971; AF028351) **100%**	0.002 ± 0.002^{a}	0 ± 0^{a}	0.792 ± 0.381^{b}	1.916 ± 0.832^{b}
OTU_307 (bacterium NLAE-zl-P408; JQ607167) **99%**	0 ± 0^{a}	0 ± 0^{a}	0.142 ± 0.063^{b}	0.002 ± 0.001^{a}
OTU_105 (Clostridium sp. BR72; KP966092) **99%**	0.002 ± 0.002^{a}	0 ± 0^{a}	2.047 ± 0.945^{b}	1.560 ± 0.554^{b}
OTU_411 (Clostridium sp. JC3; AB093546) **93%**	0.016 ± 0.007^{a}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}

3.5.3 Maternal Antibiotic/Prebiotic Exposure Impacts Body Weights and Metabolism of Their Offspring and the Effect is Stronger in Males

Different phenotypes were observed in males and females, therefore results are presented separately for males (Figure 3.5) and females (Figure 3.6). Male offspring of ABT dams had accelerated weight gain early in life (Figure 3.5A-B) despite all groups having identical caloric intake (Figure 3.5L). At weaning, ABT male offspring were heavier than any other group (Figure 3.5A) and their growth rates were increased immediately post weaning ($p=0.003$; Figure 3.5B). From weeks 5-9 of life, ABT male offspring were no longer heavier than CTR but remained heavier than PRE and ABT+PRE (Figure 3.5C). To see whether accelerated weight gain early in life increased their risk of obesity, we challenged the offspring with a HFS diet from week 9-17 of life (Figure 3.5D). After only 3 weeks on a HFS diet, ABT male offspring had gained more weight than any other group and remained heavier until the end of study (Figure 3.5D). DXA analysis revealed that greater weight gain was due to increased fat mass ($p=0.036$; Figure 3.5E) and not lean mass (Figure 3.7). Greater liver triglycerides were seen in the ABT offspring ($p<0.0001$; Figure 3.5F). At euthanasia, the ABT group had lower serum GLP-1 ($p=0.015$; Figure 3.5G) and higher fasting insulin levels ($p=0.029$; Figure 3.5I) compared to PRE and ABT+PRE. A trend toward an improvement was seen in GLP-1 (Figure 3.5G) and insulin (Figure 3.5I) when prebiotics were co-administered with antibiotics. No differences were found in leptin levels ($p=0.184$; Figure 3.5H), insulin tolerance test before (Figure 3.5J) and after (Figure 3.5K) HFS diet challenge as well as glucose tolerance tests (Figure 3.7C-D).

The observed phenotype was less evident in females (Figure 3.6A-L). ABT females had increased peri-weaning weight gain ($p=0.001$; Figure 3.6B), but was not different than CTR before weaning (Figure 3.6A) or before the HFS challenge (Figure 3.6C). After 3 weeks of HFS challenge, female ABT offspring were heavier than any other group and remained heavier until the end of the study (Figure 3.6D). The ABT group had more fat mass than the ABT+PRE group ($p=0.026$; Figure 3.6E). The PRE group had less lean mass than ABT ($p=0.047$; Figure 3.6B). There were no differences between groups in caloric intake (Figure 3.6L), fasting metabolic hormones at euthanasia (Figure 3.6G-I), insulin tolerance tests (Figure 3.6J-K) and oral glucose tolerance tests

(Figure 3.7E-F).

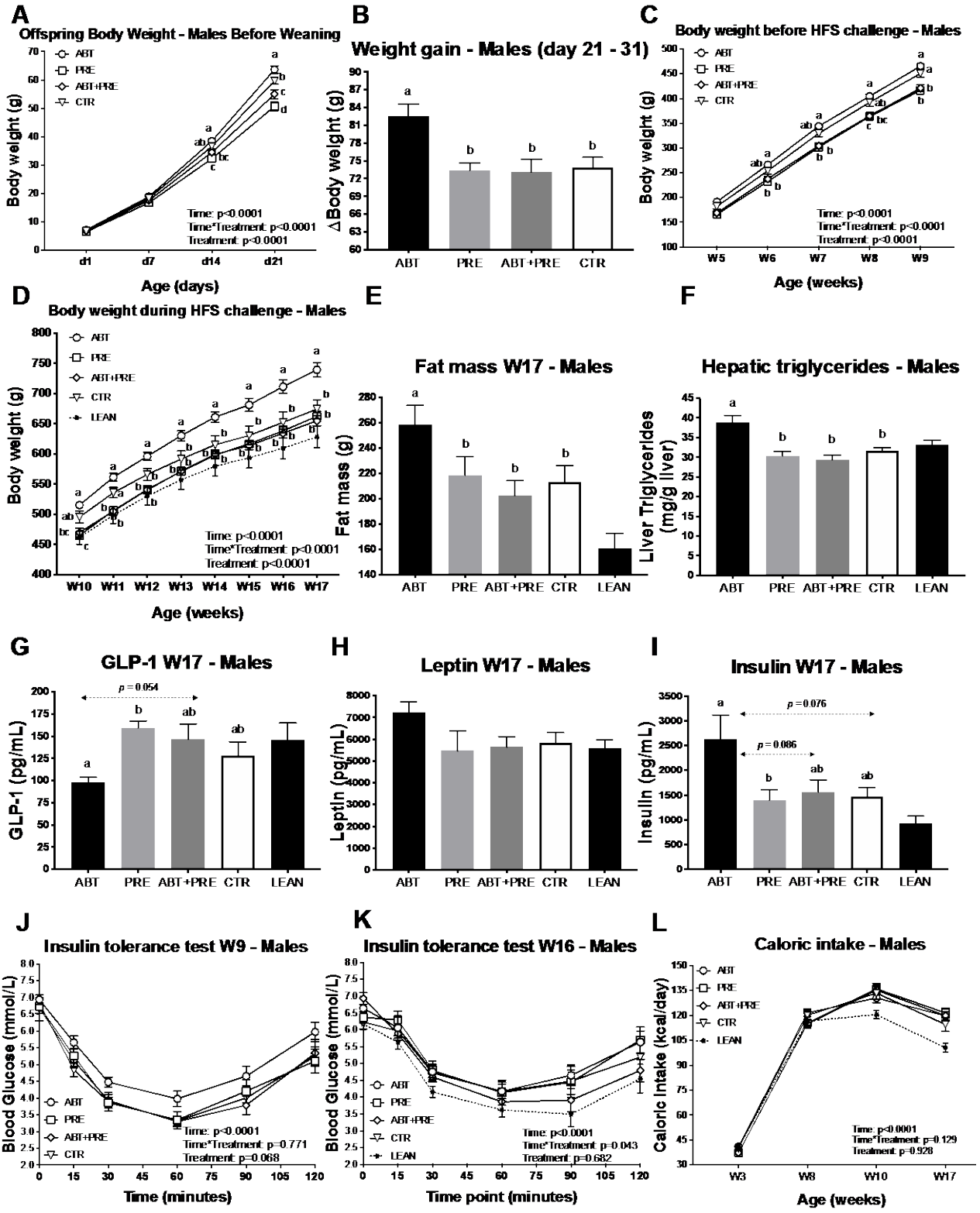

A
Offspring Body Weight - Males Before Weaning
Body weight (g)
ABT
PRE
ABT+PRE
CTR
Time: p<0.0001
Time*Treatment: p<0.0001
Treatment: p<0.0001
d1
d7
d14
d21
Age (days)
B
Weight gain - Males (day 21 - 31)
Body weight (g)
ABT
PRE
ABT+PRE
CTR
C
Body weight before HFS challenge - Males
Body weight (g)
Time: p<0.0001
Time*Treatment: p<0.0001
Treatment: p<0.0001
W5
W6
W7
W8
W9
Age (weeks)
D
Body weight during HFS challenge - Males
Body weight (g)
LEAN
Time: p<0.0001
Time*Treatment: p<0.0001
Treatment: p<0.0001
W10
W11
W12
W13
W14
W15
W16
W17
Age (weeks)
E
Fat mass W17 - Males
Fat mass (g)
LEAN
F
Hepatic triglycerides - Males
Liver Triglycerides (mg/g liver)
G
GLP-1 W17 - Males
GLP-1 (pg/mL)
p = 0.054
H
Leptin W17 - Males
Leptin (pg/mL)
I
Insulin W17 - Males
Insulin (pg/mL)
p = 0.076
p = 0.086
J
Insulin tolerance test W9 - Males
Blood Glucose (mmol/L)
Time: p<0.0001
Time*Treatment: p=0.771
Treatment: p=0.068
Time (minutes)
K
Insulin tolerance test W16 - Males
Blood Glucose (mmol/L)
Time: p<0.0001
Time*Treatment: p=0.043
Treatment: p=0.682
Time point (minutes)
L
Caloric intake - Males
Caloric Intake (kcal/day)
Time: p<0.0001
Time*Treatment: p=0.129
Treatment: p=0.928
W3
W8
W10
W17
Age (weeks)

Figure 3.5 Maternal antibiotic/prebiotic exposure impacts body weights and metabolism of their male offspring.

Body weight of male offspring before weaning (A), early-life (periweaning) growth rates (B), body weights before a HFS challenge (C) and during a HFS challenge (D) (n=7-11 rats/group). Fat mass (E) and hepatic triglycerides (F) measured at the end of the study (n=7-10 rats/group). Portal vein GLP-1 (H), leptin (I) and insulin (J) levels measured at euthanasia (n=7-10 rats/group). Glucose response during ITT before (J) and after (K) HFS challenge (n=8-11 rats/group). (L) Average caloric intake (kcal) calculated as average energy intake over 5 days measured at 4 different weeks of life (W3 – after weaning; W8 – before a HFS challenge; W10 – first week of HFS challenge; W17 – last week of HFS challenge). Results are shown as mean ± SEM. Labelled means without a common superscript letter differ ($p < 0.05$). ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; CTR, control; LEAN, lean control; d, day of life; W, week of life.

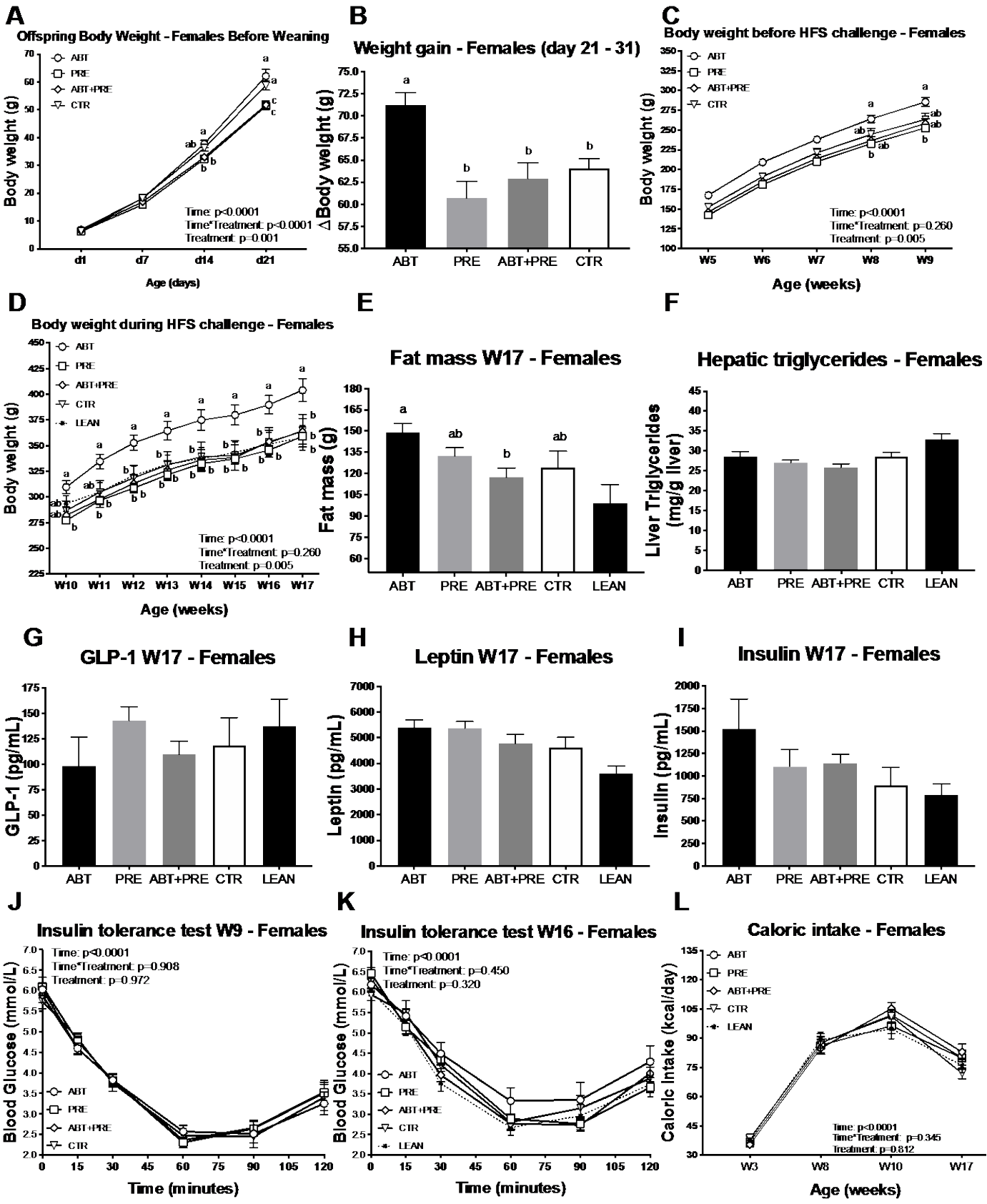

A
Offspring Body Weight - Females Before Weaning
ABT
PRE
ABT+PRE
CTR
Body weight (g)
Time: p<0.0001
Time*Treatment: p<0.0001
Treatment: p=0.001
d1
d7
d14
d21
Age (days)
B
Weight gain - Females (day 21 - 31)
ΔBody weight (g)
ABT
PRE
ABT+PRE
CTR
C
Body weight before HFS challenge - Females
Body weight (g)
Time: p<0.0001
Time*Treatment: p=0.260
Treatment: p=0.005
W5
W6
W7
W8
W9
Age (weeks)
D
Body weight during HFS challenge - Females
LEAN
Time: p<0.0001
Time*Treatment: p=0.260
Treatment: p=0.005
W10
W11
W12
W13
W14
W15
W16
W17
E
Fat mass W17 - Females
Fat mass (g)
LEAN
F
Hepatic triglycerides - Females
Liver Triglycerides (mg/g liver)
G
GLP-1 W17 - Females
GLP-1 (pg/mL)
H
Leptin W17 - Females
Leptin (pg/mL)
I
Insulin W17 - Females
Insulin (pg/mL)
J
Insulin tolerance test W9 - Females
Time: p<0.0001
Time*Treatment: p=0.908
Treatment: p=0.972
Blood Glucose (mmol/L)
Time (minutes)
K
Insulin tolerance test W16 - Females
Time: p<0.0001
Time*Treatment: p=0.450
Treatment: p=0.320
L
Caloric intake - Females
Caloric Intake (kcal/day)
Time: p<0.0001
Time*Treatment: p=0.345
Treatment: p=0.812
W3
W8
W10
W17

Figure 3.6 Maternal antibiotic/prebiotic exposure impacts body weights of their female offspring.

Body weight of male offspring before weaning (A), early-life (periweaning) growth rates (B), body weights before a HFS challenge (C) and during a HFS challenge (D) (n=7-10 rats/group). Fat mass (E) and hepatic triglycerides (F) measured at the end of the study (n=7-11 rats/group). Portal vein GLP-1 (H), leptin (I) and insulin (J) levels measured at euthanasia (n=7-10 rats/group). Glucose response during ITT before (J) and after (K) HFS challenge (n=8-10 rats/group). (L) Average caloric intake (kcal) calculated as average energy intake over 5 days measured at 4 different weeks of life (W3 – after weaning; W8 – before a HFS challenge; W10 – first week of HFS challenge; W17 – last week of HFS challenge). Results are shown as mean ± SEM. Labelled means without a common superscript letter differ ($p < 0.05$). ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; CTR, control; LEAN, lean control; d, day of life; W, week of life.

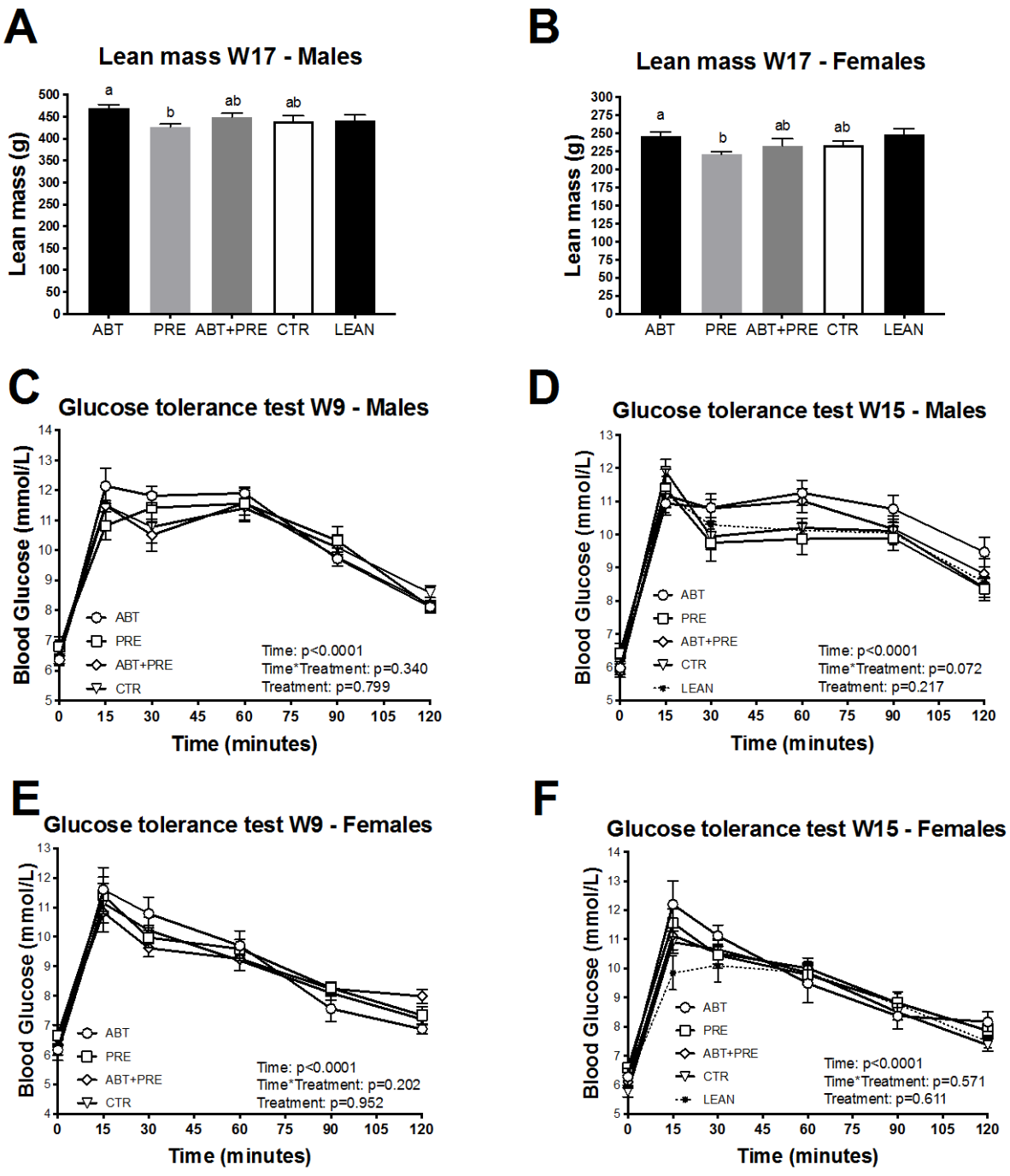


Figure 3.7 Maternal antibiotic/prebiotic exposure during pregnancy and lactation has no impact on glucose tolerance and lean mass in offspring.

Lean mass in males (A) and females (B) measured at the end of the study (n=7-11 rats/group). (C-D) OGTT in males before and after metabolic challenge (n=8-11 rats/group). (E-F) OGTT in females before and after metabolic challenge (n=8-10 rats/group). Results are

shown as mean ± SEM. Labelled means without a common superscript letter differ ($p < 0.05$). ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; CTR, control; LEAN, lean control.

3.5.4 Maternal Antibiotic/Prebiotic Exposure During Pregnancy and Lactation Impacts Microbial Profiles of Their Offspring

At the end of the study, cecal content was collected and sequenced using Illumina 16S rRNA sequencing. In male offspring (Figure 3.8A-D), no differences were seen in alpha-diversity using Chao1 (Figure 3.8C) and Shannon index (Figure 3.8D). Likewise, beta-diversity analysis (Figure 3.8B) showed most groups clustering together. The treatment explained 16.6% of the change in the bacterial community structure, likely due to the differences between the lean group and the other groups. There were no differences between groups in relative abundance at the family level (Figure 3.8A). Similarly, at the OTU-level, the lean control differed from the rest of the groups, revealing once again the strong effect of the 8-week HFS diet on the microbiota composition, likely masking other differences between groups (Table 3.3). Specifically, higher relative abundances of OTU_21 (*Ruminococcus bromii*; X85099), OTU_74 (Clostridiales bacterium CIEAF 020; AB702928), OTU_81 (*Lachnospiraceae bacterium* 605; AB700364), OTU_418 (*Clostridium* sp.; LIP5; Y12289) and OTU_882 (*Lachnospiraceae bacterium* G11; KC143064) were seen in the lean control group compared to any other group.

Similar to males, no differences in beta and alpha-diversity (Figure 3.9B-D) were seen in female offspring. Treatment explained 16.7% of the variation in the bacterial community structure (Figure 3.9B). Relative abundance on a family level showed no differences between groups (Figure 3.9A) and analysis at the OTU level (Table 3.4) showed mostly differences in the lean control group compared to any other group (Table 3.4).

While cecal microbiota at the end of the experiment did not differ substantially across treatment groups, longitudinal fecal microbiota analysis of earlier time points showed notable differences across groups. Feces were collected repeatedly in the offspring (males - Figure 3.8E-J and Figure 3.3I-O; females Figure 3.9E-J and Figure 3.3P-V). qPCR analysis of selected bacterial taxa revealed important differences between

groups chiefly immediately after weaning in males and females (week 3). Similarly to dams (Figure 3.2E), ABT male offspring had lower *Lactobacillus* spp. abundance after weaning compared to all other groups (Figure 3.8E) and higher *Enterobacteriaceae* compared to the CTR group only (Figure 3.8J). The ABT group also had higher abundance of *Clostridium coccoides* (Cluster XIV) at weaning compared to all other groups (Figure 3.8F) and *Clostridium* cluster XI at the end of study compared to all groups (Figure 3.8G). Prebiotic groups (PRE, ABT+PRE) had higher abundance of *Collinsella aerofaciens* (Figure 3.8H) and *Bifidobacterium* spp. (Figure 3.8I) than ABT and CTR immediately after weaning.

In females, lower *Lactobacillus* spp. levels after weaning were seen in ABT offspring compared to CTR (Figure 3.9E) and higher *Enterobacteriaceae* compared to all other groups (Figure 3.9J). Similar to males, ABT female offspring had higher levels of *Clostridium coccoides* (Cluster XIV) at weaning compared to all other groups (Figure 3.9F), but no differences were seen in *Clostridium* cluster XI levels at any time point between groups (Figure 3.9G). Similar to the dams, prebiotics (PRE, ABT+PRE) increased *Collinsella aerofaciens* (Figure 3.9H) and *Bifidobacterium* spp. (Figure 3.9I) compared to all other groups immediately after weaning.

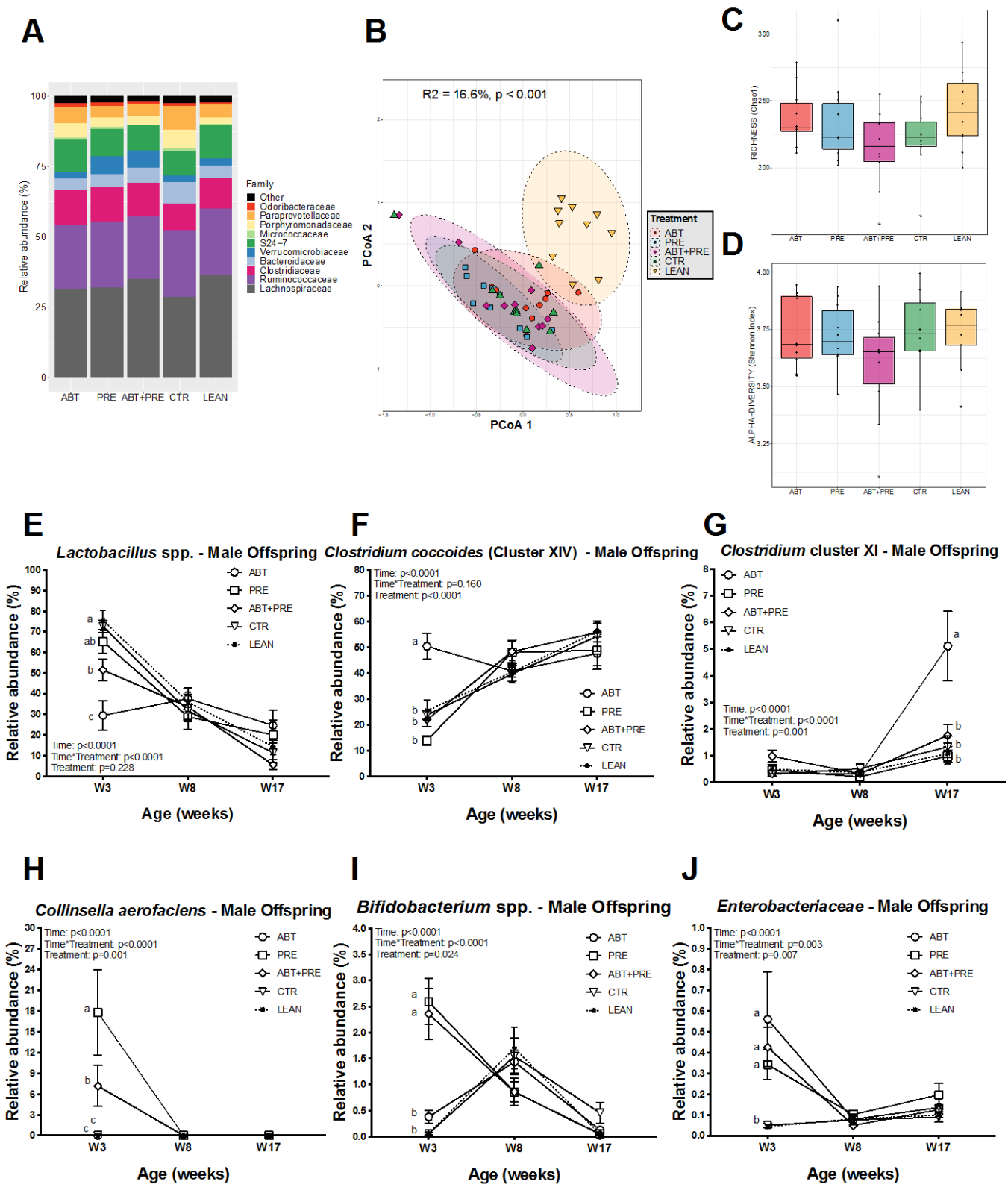

A
Relative abundance (%)
Family
Other
Odoribacteraceae
Paraprevotellaceae
Porphyromonadaceae
Micrococcaceae
S24−7
Verrucomicrobiaceae
Bacteroidaceae
Clostridiaceae
Ruminococcaceae
Lachnospiraceae
ABT PRE ABT+PRE CTR LEAN
B
R2 = 16.6%, p < 0.001
PCoA 2
PCoA 1
Treatment
ABT
PRE
ABT+PRE
CTR
LEAN
C
RICHNESS (Chao1)
D
ALPHA-DIVERSITY (Shannon Index)
E
Lactobacillus spp. - Male Offspring
Relative abundance (%)
Time: p<0.0001
Time*Treatment: p<0.0001
Treatment: p=0.228
W3 W8 W17
Age (weeks)
F
Clostridium coccoides (Cluster XIV) - Male Offspring
Time: p<0.0001
Time*Treatment: p=0.160
Treatment: p<0.0001
G
Clostridium cluster XI - Male Offspring
Time: p<0.0001
Time*Treatment: p<0.0001
Treatment: p=0.001
H
Collinsella aerofaciens - Male Offspring
Time: p<0.0001
Time*Treatment: p<0.0001
Treatment: p=0.001
I
Bifidobacterium spp. - Male Offspring
Time: p<0.0001
Time*Treatment: p<0.0001
Treatment: p=0.024
J
Enterobacteriaceae - Male Offspring
Time: p<0.0001
Time*Treatment: p=0.003
Treatment: p=0.007

Figure 3.8 Maternal antibiotic/prebiotic exposure during pregnancy and lactation impacts microbial profiles of male offspring.

Cecal matter was collected at euthanasia (W17) and sequenced using 16S rRNA Illumina sequencing. (A) Relative abundance of the ten most abundant bacterial families in male offspring (n=10 rats/group). (B) Principal-component analysis (PCoA) ordination of variation in beta-diversity of gut bacterial communities based on Bray-Curtis dissimilarities among cecal matter samples (n=10 rats/group). Chao1 estimated richness (C) and Shannon Index (D) display differences in alpha-diversity between groups (n=10 rats/group). Fecal samples were collected repeatedly (W3 - after weaning; W8 - before HFS challenge and W17 – end of study) and quantified with qPCR. Results are presented as relative abundance (%) for (E) *Lactobacillus* spp., (F) *Clostridium coccoides* (Cluster XIV), (G) *Clostridium* cluster XI, (H) *Collinsella aerofaciens*, (I) *Bifidobacterium* spp. and (J) *Enterobacteriaceae* (n=7-10 rats/group). Results are shown as mean ± SEM. Labelled means without a common superscript letter differ ($p < 0.05$). ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; CTR, control; LEAN, lean control.

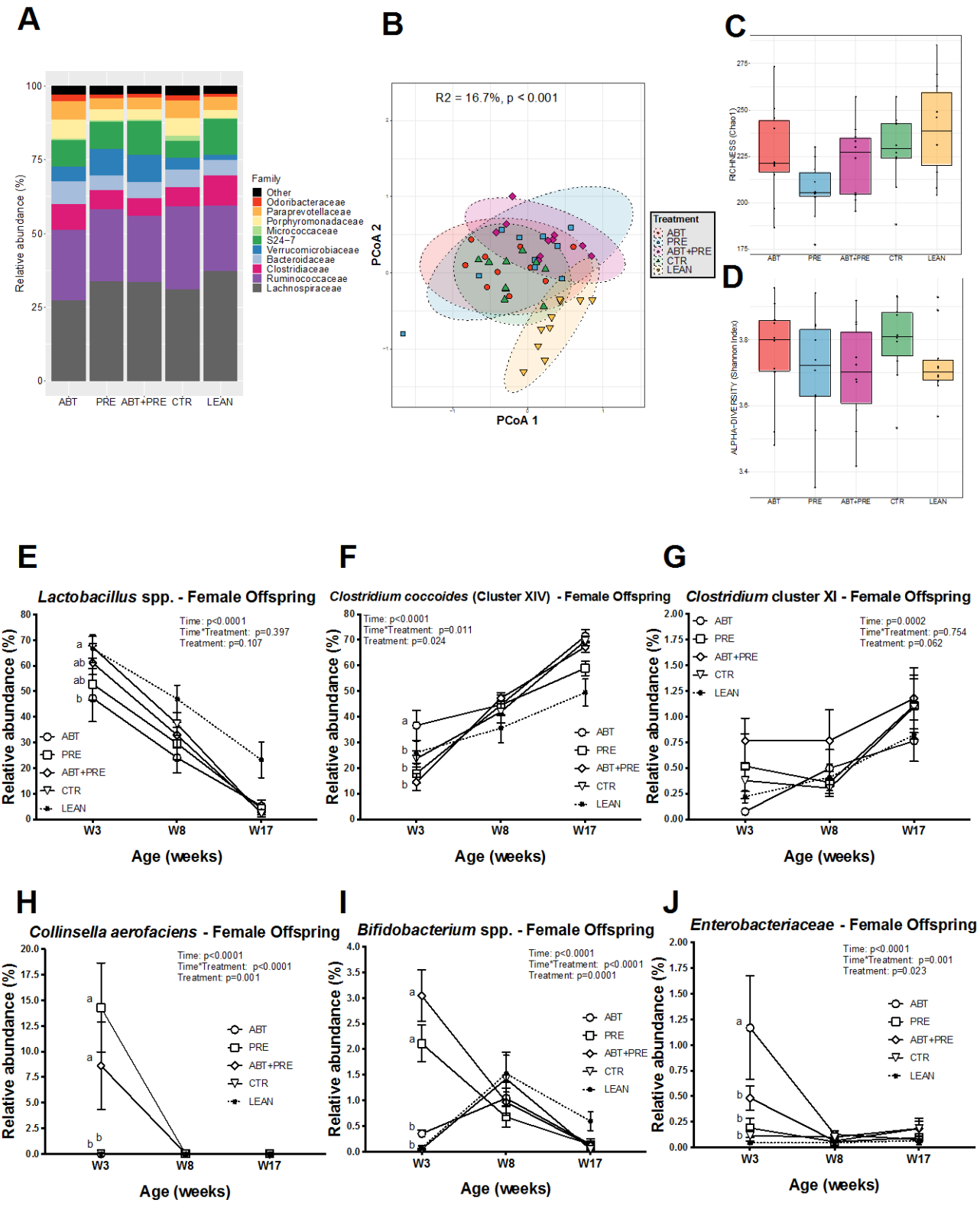

A
Relative abundance (%)
Family
Other
Odoribacteraceae
Paraprevotellaceae
Porphyromonadaceae
Micrococcaceae
S24−7
Verrucomicrobiaceae
Bacteroidaceae
Clostridiaceae
Ruminococcaceae
Lachnospiraceae
ABT PRE ABT+PRE CTR LEAN
B
R2 = 16.7%, p < 0.001
PCoA 2
PCoA 1
Treatment
ABT
PRE
ABT+PRE
CTR
LEAN
C
RICHNESS (Chao1)
D
ALPHA-DIVERSITY (Shannon Index)
E
Lactobacillus spp. - Female Offspring
Time: p<0.0001
Time*Treatment: p=0.397
Treatment: p=0.107
F
Clostridium coccoides (Cluster XIV) - Female Offspring
Time: p<0.0001
Time*Treatment: p=0.011
Treatment: p=0.024
G
Clostridium cluster XI - Female Offspring
Time: p=0.0002
Time*Treatment: p=0.754
Treatment: p=0.062
Relative abundance (%)
W3 W8 W17
Age (weeks)
H
Collinsella aerofaciens - Female Offspring
Time: p<0.0001
Time*Treatment: p<0.0001
Treatment: p=0.001
I
Bifidobacterium spp. - Female Offspring
Time: p<0.0001
Time*Treatment: p<0.0001
Treatment: p=0.0001
J
Enterobacteriaceae - Female Offspring
Time: p<0.0001
Time*Treatment: p=0.001
Treatment: p=0.023

Figure 3.9 Maternal antibiotic/prebiotic exposure during pregnancy and lactation impacts microbial profiles of female offspring.

Cecal matter was collected at euthanasia (W17) and sequenced using 16S rRNA Illumina sequencing. (A) Relative abundance of the 10 most abundant bacterial families in female offspring (n=10 rats/group). (B) Principal-component analysis (PCoA) ordination of variation in beta-diversity of gut bacterial communities based on Bray-Curtis dissimilarities among cecal matter samples (n=10 rats/group). Chao1 estimated richness (C) and Shannon Index (D) display differences in alpha-diversity between groups (n=10 rats/group). Fecal samples were collected repeatedly (W3 - after weaning; W8 - before HFS challenge and W17 – end of study) and quantified with qPCR. Results are presented as relative abundance (%) for (E) *Lactobacillus* spp., (F) *Clostridium coccoides* (Cluster XIV), (G) *Clostridium* cluster XI, (H) *Collinsella aerofaciens*, (I) *Bifidobacterium* spp. and (J) *Enterobacteriaceae* (n=7-10 rats/group). Results are shown as mean ± SEM. Labelled means without a common superscript letter differ ($p < 0.05$). ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; CTR, control; LEAN, lean control.

Table 3.3 Relative Abundance of Bacterial Taxa in Ceca of Male Offspring at the End of Study Using Illumina 16S rRNA Gene Sequencing.

[P value (adj. val.), FDR significance set at 0.05; n=10 rats/group. OTU, operational taxonomic units.]

Treatment	Control	Antibiotic	Prebiotic	Antibiotic+Prebiotic	Lean Control
Phyla					
Bacteroidetes	23.328 ± 1.042^{b}	$20.487 \pm 2.050^{a,b}$	16.780 ± 2.031^{a}	16.452 ± 1.253^{a}	18.480 ± 1.269^{a}
Actinobacteria	0.846 ± 0.119^{b}	$0.544 \pm 0.080^{a,b}$	$0.659 \pm 0.165^{a,b}$	0.365 ± 0.046^{a}	$0.633 \pm 0.141^{a,b}$
Deferribacteres	0.040 ± 0.014^{a}	0.046 ± 0.019^{a}	0 ± 0^{b}	0.005 ± 0.005^{b}	0.057 ± 0.022^{a}
Class					
Erysipelotrichia	$0.278 \pm 0.082^{a,b}$	0.368 ± 0.082^{a}	0.106 ± 0.032^{b}	$0.261 \pm 0.096^{a,b}$	0.344 ± 0.041^{a}
Bacteroidia	23.293 ± 1.040^{b}	$20.454 \pm 2.050^{a,b}$	16.753 ± 2.030^{a}	16.433 ± 1.254^{a}	18.445 ± 1.264^{a}
Actinobacteria	0.846 ± 0.119^{b}	$0.544 \pm 0.080^{a,b}$	$0.659 \pm 0.165^{a,b}$	0.365 ± 0.046^{a}	$0.633 \pm 0.141^{a,b}$
Deferribacteres	0.040 ± 0.014^{a}	0.046 ± 0.019^{a}	0 ± 0^{b}	0.005 ± 0.005^{b}	0.057 ± 0.022^{a}
Order					
Erysipelotrichales	$0.278 \pm 0.082^{a,b}$	0.368 ± 0.082^{a}	0.106 ± 0.032^{b}	$0.261 \pm 0.096^{a,b}$	0.344 ± 0.041^{a}
Bacteroidales	23.293 ± 1.040^{b}	$20.454 \pm 2.050^{a,b}$	16.753 ± 2.030^{a}	16.433 ± 1.254^{a}	18.445 ± 1.264^{a}
unclassified_"Bacteroidales"	0.665 ± 0.067^{a}	0.664 ± 0.072^{a}	0.532 ± 0.059^{a}	0.357 ± 0.042^{b}	0.331 ± 0.043^{b}
Rhodospirillales	0.051 ± 0.026^{b}	$0.017 \pm 0.007^{a,b}$	0.003 ± 0.002^{a}	$0.020 \pm 0.010^{a,b}$	$0.017 \pm 0.007^{a,b}$
Actinomycetales	0.651 ± 0.118^{b}	$0.309 \pm 0.073^{a,b}$	$0.476 \pm 0.167^{a,b}$	0.235 ± 0.048^{a}	0.259 ± 0.049^{a}
Micrococcineae (suborder)	0.651 ± 0.118^{b}	$0.308 \pm 0.072^{a,b}$	$0.474 \pm 0.167^{a,b}$	0.234 ± 0.048^{a}	0.257 ± 0.049^{a}
Bifidobacteriales	0.006 ± 0.003^{a}	$0.034 \pm 0.027^{a,b}$	0.004 ± 0.002^{a}	0.008 ± 0.002^{a}	0.262 ± 0.137^{b}
Deferribacterales	0.040 ± 0.014^{a}	0.046 ± 0.019^{a}	0 ± 0^{b}	0.005 ± 0.005^{b}	0.057 ± 0.022^{a}
Family					
Erysipelotrichaceae	$0.278 \pm 0.082^{a,b}$	0.368 ± 0.082^{a}	0.106 ± 0.032^{b}	$0.261 \pm 0.096^{a,b}$	0.344 ± 0.041^{a}
Streptococcaceae	$0.122 \pm 0.019^{a,c}$	$0.079 \pm 0.009^{a,c}$	0.125 ± 0.017^{c}	$0.062 \pm 0.008^{a,b}$	0.037 ± 0.009^{b}
Prevotellaceae	6.077 ± 0.481^{b}	$4.526 \pm 0.858^{a,b}$	2.994 ± 0.657^{a}	3.102 ± 0.708^{a}	3.720 ± 0.493^{a}
unclassified_"Prevotellaceae"	0.009 ± 0.002^{b}	$0.014 \pm 0.005^{a,b}$	$0.004 \pm 0.001^{a,b}$	0.004 ± 0.004^{a}	$0.007 \pm 0.003^{a,b}$

Micrococcaceae	0.645 ± 0.118^{b}	$0.303 \pm 0.071^{a,b}$	$0.465 \pm 0.165^{a,b}$	0.232 ± 0.048^{a}	0.247 ± 0.048^{a}
Bifidobacteriaceae	0.006 ± 0.003^{a}	$0.034 \pm 0.027^{a,b}$	0.004 ± 0.002^{a}	0.008 ± 0.002^{a}	0.262 ± 0.137^{b}
unclassified_Bifidobacteriaceae	0 ± 0^{a}	0.003 ± 0.003^{a}	0 ± 0^{a}	0 ± 0^{a}	0.017 ± 0.008^{b}
Deferribacteraceae	0.040 ± 0.014^{a}	0.046 ± 0.019^{a}	0 ± 0^{b}	0.005 ± 0.005^{b}	0.057 ± 0.022^{a}
Genus					
Lachnospiracea_incertae_sedis	$0.564 \pm 0.142^{a,b}$	0.280 ± 0.100^{a}	0.327 ± 0.128^{a}	0.903 ± 0.154^{b}	$0.553 \pm 0.092^{a,b}$
Acetatifactor	1.534 ± 0.335^{b}	1.666 ± 0.595^{b}	1.722 ± 0.241^{b}	1.426 ± 0.333^{b}	0.508 ± 0.079^{a}
Flavonifractor	1.109 ± 0.126^{a}	1.041 ± 0.124^{a}	1.545 ± 0.133^{b}	$1.129 \pm 0.132^{a,b}$	0.979 ± 0.100^{a}
Pseudoflavonifractor	0.539 ± 0.154^{a}	0.419 ± 0.093^{a}	0.487 ± 0.116^{a}	$0.337 \pm 0.092^{a,b}$	0.139 ± 0.025^{b}
Terrisporobacter	0.771 ± 0.311^{a}	1.129 ± 0.281^{a}	0.664 ± 0.179^{a}	1.144 ± 0.251^{a}	0.226 ± 0.180^{b}
Anaerovorax	0.009 ± 0.003^{a}	$0.005 \pm 0.003^{a,b}$	0.001 ± 0.001^{b}	0.001 ± 0.001^{b}	$0.008 \pm 0.004^{a,b}$
Clostridium XVIII	$0.201 \pm 0.072^{a,b}$	0.250 ± 0.066^{a}	0.038 ± 0.027^{b}	$0.194 \pm 0.103^{a,b}$	0.178 ± 0.039^{a}
Turicibacter	0.012 ± 0.005^{a}	0.030 ± 0.011^{a}	0.028 ± 0.009^{a}	0.020 ± 0.006^{a}	0.121 ± 0.032^{b}
Lactococcus	0.060 ± 0.007^{a}	$0.050 \pm 0.009^{a,c}$	0.076 ± 0.010^{a}	0.035 ± 0.006^{c}	0 ± 0^{b}
Parabacteroides	4.543 ± 0.718^{b}	$3.608 \pm 0.759^{a,b}$	2.388 ± 0.843^{a}	2.276 ± 0.466^{a}	1.916 ± 0.320^{a}
Barnesiella	0.267 ± 0.088^{a}	0.471 ± 0.083^{a}	0.507 ± 0.117^{a}	0.454 ± 0.070^{a}	2.138 ± 0.367^{b}
Alloprevotella	5.805 ± 0.483^{b}	$4.234 \pm 0.856^{a,b}$	2.721 ± 0.634^{a}	2.858 ± 0.661^{a}	3.368 ± 0.396^{a}
Rothia	0.642 ± 0.118^{b}	$0.301 \pm 0.070^{a,b}$	$0.465 \pm 0.164^{a,b}$	0.231 ± 0.048^{a}	0.246 ± 0.048^{a}
Bifidobacterium	0.006 ± 0.003^{a}	$0.032 \pm 0.024^{a,b}$	0.004 ± 0.002^{a}	0.008 ± 0.002^{a}	0.244 ± 0.129^{b}
Enterorhabdus	0.101 ± 0.020^{a}	0.117 ± 0.025^{a}	0.101 ± 0.017^{a}	$0.069 \pm 0.013^{a,b}$	0.041 ± 0.008^{b}
Mucispirillum	0.040 ± 0.014^{a}	0.046 ± 0.019^{a}	0 ± 0^{b}	0.005 ± 0.005^{b}	0.057 ± 0.022^{a}
unclassified_Bacteria	$1.238 \pm 0.124^{a,b}$	1.344 ± 0.098^{a}	$1.046 \pm 0.080^{a,b}$	0.835 ± 0.068^{b}	1.309 ± 0.127^{a}
unclassified_Root	0.161 ± 0.022^{a}	$0.103 \pm 0.015^{a,b}$	0.186 ± 0.033^{a}	0.064 ± 0.007^{b}	$0.133 \pm 0.021^{a,b}$
OTUs					
OTU_21 (Ruminococcus bromii; X85099) **97%**	0 ± 0^{a}	0 ± 0^{a}	0.002 ± 0.002^{a}	0.002 ± 0.001^{a}	3.486 ± 0.837^{b}
OTU_5 (Bacteroides sp. ASF519; ASF 519; AF157056) **100%**	5.655 ± 0.896^{a}	$4.485 \pm 0.961^{a,b}$	3.067 ± 1.107^{b}	2.795 ± 0.525^{b}	2.235 ± 0.389^{b}
OTU_24 (Gram-negative bacterium cL10-2b-4; AY239469) **89%**	0.154 ± 0.045^{a}	0.072 ± 0.189^{a}	0.134 ± 0.050^{a}	0.104 ± 0.052^{a}	2.494 ± 0.456^{b}
OTU_12 (Bifidobacterium choerinum; 7 VI A; JQ002525) **99%**	0.008 ± 0.004^{a}	$0.045 \pm 0.035^{a,b}$	0.006 ± 0.003^{a}	$0.010 \pm 0.003^{a,b}$	0.295 ± 0.157^{b}

OTU_74 (Clostridiales bacterium CIEAF 020; AB702928) **97%**	0.101 ± 0.044^{a}	0.058 ± 0.029^{a}	0.707 ± 0.371^{a}	0.042 ± 0.018^{a}	0.893 ± 0.147^{b}
OTU_44 (Terrisporobacter glycolicus; 2002-87768; AY244773) **100%**	$3.626 \pm 0.836^{a,b}$	4.037 ± 0.449^{a}	$3.423 \pm 0.462^{a,b}$	3.944 ± 0.509^{a}	1.812 ± 0.251^{b}
OTU_43 (Rothia sp. EGY-WPhB4; KJ545601) **98%**	0.805 ± 0.145^{a}	$0.387 \pm 0.092^{a,b}$	$0.603 \pm 0.217^{a,b}$	$0.289 \pm 0.055^{a,b}$	0.299 ± 0.059^{b}
OTU_81 (Lachnospiraceae bacterium 605; AB700364) **99%**	0.021 ± 0.008^{a}	0.033 ± 0.006^{a}	0.028 ± 0.010^{a}	0.034 ± 0.014^{a}	1.199 ± 0.235^{b}
OTU_291 (Catabacter hongkongensis (T); HKU16; AY574991) **92%**	$0.013 \pm 0.003^{a,b}$	0.021 ± 0.004^{a}	$0.009 \pm 0.004^{a,b}$	0.004 ± 0.002^{b}	0.003 ± 0.001^{b}
OTU_25 (Clostridium sp. Culture-1; AB622814) **99%**	$2.488 \pm 0.259^{a,b}$	$2.428 \pm 0.280^{a,b}$	3.544 ± 0.331^{a}	$2.482 \pm 0.264^{a,b}$	1.860 ± 0.133^{b}
OTU_160 (bacterium YE57; AY442821) **97%**	0.021 ± 0.005^{a}	0.017 ± 0.007^{a}	0.014 ± 0.004^{a}	0.015 ± 0.003^{a}	0.060 ± 0.006^{b}
OTU_980 (Murimonas intestini (T); SRB530; KC311366) **97%**	0 ± 0^{a}	$0.003 \pm 0.003^{a,b}$	0 ± 0^{a}	0 ± 0^{a}	0.013 ± 0.006^{b}
OTU_32 (Gram-negative bacterium cL10-2b-4; AY239469) **90%**	1.141 ± 0.122^{a}	1.109 ± 0.086^{a}	0.976 ± 0.111^{a}	0.823 ± 0.090^{a}	0.510 ± 0.048^{b}
OTU_153 (Clostridium sp. Clone-7; AB622834) **97%**	0.013 ± 0.004^{b}	$0.058 \pm 0.31^{a,b}$	$0.090 \pm 0.038^{a,b}$	0.079 ± 0.030^{a}	0.108 ± 0.026^{a}
OTU_253 (Turicibacter sp. LA61; AB727348) **100%**	0.006 ± 0.004^{a}	0.009 ± 0.005^{a}	$0.013 \pm 0.005^{a,b}$	$0.013 \pm 0.004^{a,b}$	0.047 ± 0.013^{b}
OTU_60 (Roseburia sp. 499; JX629259) **97%**	$0.301 \pm 0.106^{a,b}$	0.612 ± 0.164^{a}	$0.028 \pm 0.014^{b,c}$	0.018 ± 0.012^{c}	$0.299 \pm 0.130^{a,c}$
OTU_41 (Ruminococcus sp. DJF_VR67; EU728791) **97%**	$0.246 \pm 0.162^{a,b}$	$0.387 \pm 0.225^{a,b}$	0.029 ± 0.028^{a}	$0.045 \pm 0.027^{a,b}$	0.627 ± 0.176^{b}
OTU_53 (Gram-negative bacterium cTPY-13; AY239461) **84%**	0.629 ± 0.083^{a}	0.668 ± 0.075^{a}	$0.578 \pm 0.078^{a,c}$	0.373 ± 0.058^{c}	0.217 ± 0.031^{c}
OTU_22 (Clostridium sp. A9; DQ789119) **98%**	3.010 ± 0.276^{a}	2.652 ± 0.377^{a}	$2.208 \pm 0.247^{a,b}$	2.402 ± 0.252^{a}	1.627 ± 0.178^{b}
OTU50 - Intestinimonas sp. FSAA-17; KP114242 **96%**	0.855 ± 0.135^{a}	$0.618 \pm 0.083^{a,b}$	0.659 ± 0.108^{a}	$0.627 \pm 0.239^{a,b}$	0.369 ± 0.110^{b}
OTU_79 (Clostridiales bacterium CIEAF 015; AB702929) **93%**	0.400 ± 0.074^{a}	$0.292 \pm 0.104^{a,b}$	0.626 ± 0.120^{a}	0.541 ± 0.200^{a}	0.113 ± 0.032^{b}
OTU_419 (Clostridium sp. BPY5; KM360180) **95%**	$0.003 \pm 0.003^{a,b}$	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0.005 ± 0.002^{b}

OTU_265 (Clostridiales bacterium CIEAF 015; AB702929) **93%**	$0.226 \pm 0.045^{a,b}$	$0.170 \pm 0.080^{a,b}$	0.394 ± 0.078^{a}	$0.297 \pm 0.136^{a,b}$	0.089 ± 0.026^{b}
OTU_142 (Aestuariispira insulae (T); AH-MY2; KF876014) **86%**	0.064 ± 0.034^{a}	$0.015 \pm 0.009^{a,b}$	0 ± 0^{b}	$0.025 \pm 0.014^{a,b}$	$0.012 \pm 0.007^{a,b}$
OTU_69 (Lachnospiraceae bacterium 19gly4; AF550610) **94%**	0.568 ± 0.153^{a}	0.946 ± 0.420^{a}	0.751 ± 0.189^{a}	0.577 ± 0.151^{a}	0.009 ± 0.007^{b}
OTU_214 (Lactobacillus murinus; ONS2; AY324630) **100%**	$0.032 \pm 0.009^{a,b}$	0.018 ± 0.005^{a}	0.014 ± 0.007^{a}	0.025 ± 0.017^{a}	0.096 ± 0.025^{b}
OTU_133 (Kocuria sp. SA14; KJ599867) **81%**	$0.046 \pm 0.013^{a,b}$	0.071 ± 0.010^{a}	0.020 ± 0.006^{b}	$0.045 \pm 0.012^{a,b}$	$0.043 \pm 0.012^{a,b}$
OTU_418 (Clostridium sp.; LIP5; Y12289) **96%**	0.001 ± 0.001^{a}	0 ± 0^{a}	0.076 ± 0.075^{a}	0 ± 0^{a}	0.164 ± 0.048^{b}
OTU_861 (Candidatus Soleaferrea massiliensis AP7; JX101688) **94%**	0.015 ± 0.007^{a}	0.013 ± 0.005^{a}	0.003 ± 0.003^{a}	0 ± 0^{b}	0 ± 0^{b}
OTU_2 (Alloprevotella rava; F0323; GU470887) **87%**	7.417 ± 0.660^{a}	$5.456 \pm 1.060^{a,b}$	3.566 ± 0.854^{b}	3.781 ± 0.888^{b}	4.099 ± 0.465^{b}
OTU_233 (Enterococcus durans (T); DSM20633; AJ276354) **100%**	0.033 ± 0.015^{a}	0.022 ± 0.010^{a}	0.019 ± 0.008^{a}	$0.023 \pm 0.013^{a,b}$	0 ± 0^{b}
OTU_340 (Clostridium sp. Culture Jar-13; AB622826) **87%**	0 ± 0^{a}	0 ± 0^{a}	$0.019 \pm 0.010^{a,b}$	0.018 ± 0.008^{b}	0 ± 0^{a}
OTU_754 (Clostridiales bacterium CIEAF 030; AB702933) **95%**	$0.009 \pm 0.005^{a,b}$	0.010 ± 0.005^{a}	$0.003 \pm 0.002^{a,b}$	0 ± 0^{b}	$0.005 \pm 0.002^{a,b}$
OTU_342 (Peptostreptococcaceae bacterium canine oral taxon 221; PV088; JN713384) **95%**	0.009 ± 0.003^{a}	0.009 ± 0.005^{a}	0 ± 0^{b}	0 ± 0^{b}	0.009 ± 0.004^{a}
OTU_232 (Clostridiales bacterium canine oral taxon 219; PV085; JN713382) **91%**	0 ± 0^{a}	$0.015 \pm 0.008^{a,b}$	0.001 ± 0.001^{a}	0 ± 0^{a}	0.052 ± 0.017^{b}
OTU_217 (Anaerotruncus sp. MT15; LN881593) **93%**	0.017 ± 0.003^{a}	0.023 ± 0.004^{a}	$0.021 \pm 0.006^{a,b}$	0.028 ± 0.007^{a}	0.007 ± 0.004^{b}
OTU_1039 (Lachnospiraceae bacterium 607; AB700365) **95%**	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0.005 ± 0.002^{b}
OTU_286 (Denitrobacterium detoxificans; NPOH3; AF079506) **89%**	0.004 ± 0.002^{a}	0.011 ± 0.005^{a}	0 ± 0^{b}	0 ± 0^{b}	0.014 ± 0.004^{a}
OTU_882 (Lachnospiraceae bacterium G11; KC143064) **96%**	0.001 ± 0.001^{a}	$0.004 \pm 0.002^{a,c}$	0.009 ± 0.002^{c}	$0.005 \pm 0.003^{a,c}$	0.777 ± 0.312^{b}

OTU_509 (Ruminococcus gnavus (T); ATCC 29149; X94967) **99%**	$0.004 \pm 0.002^{a,b}$	0.006 ± 0.001^{a}	$0.005 \pm 0.002^{a,b}$	$0.003 \pm 0.003^{a,b}$	0 ± 0^{b}
OTU_102 (Lachnospiraceae bacterium A4; DQ789118) **99%**	0.522 ± 0.089^{a}	$0.314 \pm 0.050^{a,b}$	$0.513 \pm 0.128^{a,b}$	0.507 ± 0.083^{a}	0.195 ± 0.057^{b}
OTU_213 (Clostridium thermocellum; L09173) **91%**	0 ± 0^{a}	$0.025 \pm 0.022^{a,b}$	$0.021 \pm 0.018^{a,b}$	0.003 ± 0.002^{a}	0.061 ± 0.028^{b}
OTU_154 (Peptostreptococcaceae bacterium canine oral taxon 019; OB001; JN713180) **86%**	0.015 ± 0.004^{a}	0.014 ± 0.007^{a}	0.008 ± 0.004^{a}	0.024 ± 0.008^{a}	0.139 ± 0.027^{b}
OTU_481 (Clostridium sp. BMD1; HG931333) **96%**	$0.375 \pm 0.099^{a,b}$	$0.367 \pm 0.184^{a,b}$	0.826 ± 0.160^{a}	0.528 ± 0.186^{a}	0.131 ± 0.013^{b}
OTU_66 (Roseburia intestinalis (T); L1-82; AJ312385) **93%**	$0.299 \pm 0.066^{a,b}$	$0.158 \pm 0.051^{a,c}$	0.509 ± 0.110^{b}	$0.279 \pm 0.106^{a,b}$	0.056 ± 0.028^{c}
OTU_169 (Ruminiclostridium thermocellum; HAW3/1; HG917913) **94%**	0.004 ± 0.003^{a}	$0.011 \pm 0.004^{a,b}$	0.081 ± 0.030^{b}	0.014 ± 0.013^{a}	0.047 ± 0.018^{b}
OTU_266 (Lactococcus lactis; UK1560; from a traditional fermented milk; AJ271851) **100%**	$0.029 \pm 0.004^{a,c}$	$0.026 \pm 0.003^{a,c}$	0.046 ± 0.007^{c}	0.022 ± 0.004^{a}	0 ± 0^{b}
OTU_127 (Enterorhabdus caecimuris (T); B7; DQ789120) **98%**	0.012 ± 0.026^{a}	0.137 ± 0.029^{a}	0.117 ± 0.020^{a}	$0.085 \pm 0.017^{a,b}$	0.043 ± 0.010^{b}
OTU_246 (Clostridiales bacterium CIEAF 015; AB702929) **91%**	0.017 ± 0.007^{a}	$0.003 \pm 0.002^{a,b}$	0.021 ± 0.008^{a}	0.050 ± 0.026^{a}	0 ± 0^{b}
OTU_313 (Clostridium sp. SL-2013-71; HG326494) **92%**	0.001 ± 0.001^{a}	$0.008 \pm 0.003^{a,b}$	$0.007 \pm 0.004^{a,b}$	0.019 ± 0.008^{b}	0.001 ± 0.001^{a}
OTU_474 (Streptococcus mutans; UA140; AF139599) **100%**	$0.004 \pm 0.002^{a,b}$	0 ± 0^{a}	$0.006 \pm 0.003^{a,b}$	0 ± 0^{a}	0.009 ± 0.003^{b}
OTU_187 (Clostridiales bacterium oral taxon F32; VO026; HM099644) **92%**	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0.204 ± 0.135^{b}

Table 3.4 Relative Abundance of Bacterial Taxa in Ceca of Female Offspring at the End of Study Using Illumina 16S rRNA Gene Sequencing.

[P value (adj. val.), FDR significance set at 0.05; n=10 rats/group. OTU, operational taxonomic units.]

Treatment	Control	Antibiotic	Prebiotic	Antibiotic+Prebiotic	Lean Control
Phyla					
Verrucomicrobia(A vs P p=0.10; A vs AP p= 0.07 C vs P p=0.07; C vs AP p = 0.07)	$2.864 \pm 0.959^{a,b}$	$3.357 \pm 0.975^{a,b}$	6.507 ± 1.394^{b}	6.505 ± 0.775^{b}	1.433 ± 0.401^{a}
unclassified_Firmicutes	$0.386 \pm 0.061^{a,b}$	0.243 ± 0.020^{a}	$0.364 \pm 0.054^{a,b}$	$0.381 \pm 0.080^{a,b}$	0.690 ± 0.089^{b}
Deferribacteres	0.059 ± 0.018^{a}	0.057 ± 0.013^{a}	0 ± 0^{b}	0 ± 0^{b}	0.061 ± 0.012^{a}
Class					
Verrucomicrobiae (A vs P p=0.10; A vs AP p= 0.07 C vs P p=0.07; C vs AP p = 0.07)	$2.864 \pm 0.959^{a,b}$	$3.357 \pm 0.975^{a,b}$	6.506 ± 1.394^{b}	6.505 ± 0.775^{b}	1.433 ± 0.401^{a}
Deferribacteres	0.059 ± 0.018^{a}	0.057 ± 0.013^{a}	0 ± 0^{b}	0 ± 0^{b}	0.061 ± 0.012^{a}
Order					
Verrucomicrobiales (A vs P p=0.10; A vs AP p= 0.07 C vs P p=0.07; C vs AP p = 0.07)	$2.864 \pm 0.959^{a,b}$	$3.357 \pm 0.975^{a,b}$	6.506 ± 1.394^{b}	6.505 ± 0.775^{b}	1.433 ± 0.401^{a}
unclassified_Clostridiales_Incertae Sedis XIII	0.011 ± 0.003^{a}	$0.003 \pm 0.002^{a,b}$	$0.008 \pm 0.003^{a,b}$	0.001 ± 0.001^{b}	$0.003 \pm 0.002^{a,b}$
unclassified_Lactobacillales	$0.047 \pm 0.016^{a,b}$	0.010 ± 0.003^{a}	$0.046 \pm 0.023^{a,b}$	$0.025 \pm 0.007^{a,b}$	0.049 ± 0.011^{b}
unclassified_"Bacteroidales"	0.555 ± 0.046^{a}	0.618 ± 0.083^{a}	$0.401 \pm 0.061^{a,b}$	0.557 ± 0.053^{a}	0.345 ± 0.025^{b}
Bifidobacteriales	0.008 ± 0.005^{a}	0.004 ± 0.002^{a}	0.006 ± 0.004^{a}	0.044 ± 0.025^{a}	0.563 ± 0.211^{b}
Deferribacterales	0.059 ± 0.018^{a}	0.057 ± 0.013^{a}	0 ± 0^{b}	0 ± 0^{b}	0.061 ± 0.012^{a}
Family					
Clostridiaceae 1	2.147 ± 0.460^{a}	3.477 ± 1.675^{a}	2.455 ± 0.505^{a}	2.116 ± 0.422^{a}	6.133 ± 0.747^{b}
Lactobacillaceae	$0.061 \pm 0.025^{a,b}$	0.021 ± 0.009^{a}	0.039 ± 0.017^{a}	0.034 ± 0.017^{a}	0.140 ± 0.031^{b}
Verrucomicrobiaceae (A vs P p=0.10; A vs AP p= 0.07 C vs P p=0.07; C vs AP p = 0.07)	$2.864 \pm 0.959^{a,b}$	$3.357 \pm 0.975^{a,b}$	6.506 ± 1.394^{b}	6.505 ± 0.775^{b}	1.433 ± 0.401^{a}

Bifidobacteriaceae	0.008 ± 0.005^{a}	0.004 ± 0.002^{a}	0.006 ± 0.004^{a}	0.044 ± 0.025^{a}	0.563 ± 0.211^{b}
unclassified_Bifidobacteriaceae	0 ± 0^{a}	0 ± 0^{a}	0.002 ± 0.001^{a}	0.001 ± 0.001^{a}	0.023 ± 0.010^{b}
Deferribacteraceae	0.059 ± 0.018^{a}	0.057 ± 0.013^{a}	0 ± 0^{b}	0 ± 0^{b}	0.061 ± 0.012^{a}
Genus					
Intestinimonas	$0.201 \pm 0.027^{a,b}$	0.279 ± 0.043^{a}	0.134 ± 0.021^{c}	$0.145 \pm 0.020^{b,c}$	$0.215 \pm 0.021^{a,b}$
Anaerotruncus	0.149 ± 0.024^{a}	0.181 ± 0.041^{a}	0.173 ± 0.033^{a}	0.194 ± 0.027^{a}	0.065 ± 0.013^{b}
Terrisporobacter	0.165 ± 0.101^{c}	$0.693 \pm 0.297^{a,c}$	0.512 ± 0.148^{a}	$0.200 \pm 0.083^{a,c}$	0.025 ± 0.022^{b}
Clostridium sensu stricto	2.117 ± 0.454^{a}	3.433 ± 1.655^{a}	2.402 ± 0.496^{a}	2.070 ± 0.411^{a}	6.044 ± 0.738^{b}
Turicibacter	0.025 ± 0.007^{a}	0.040 ± 0.032^{a}	0.034 ± 0.009^{a}	0.026 ± 0.008^{a}	0.257 ± 0.052^{b}
Lactobacillus	$0.061 \pm 0.025^{a,b}$	0.021 ± 0.009^{a}	0.039 ± 0.017^{a}	0.034 ± 0.017^{a}	0.140 ± 0.031^{b}
Lactococcus	0.100 ± 0.068^{a}	0.033 ± 0.007^{a}	0.038 ± 0.010^{a}	0.044 ± 0.013^{a}	0 ± 0^{b}
Parabacteroides	4.440 ± 0.532^{a}	$4.610 \pm 0.820^{a,b}$	$2.641 \pm 0.952^{a,b}$	$2.421 \pm 0.993^{a,b}$	2.032 ± 0.368^{b}
Barnesiella	0.287 ± 0.068^{a}	0.337 ± 0.118^{a}	0.461 ± 0.124^{a}	0.634 ± 0.172^{a}	2.019 ± 0.253^{b}
Akkermansia (A vs P p=0.10; A vs AP p= 0.07 C vs P p=0.07; C vs AP p = 0.07)	$2.864 \pm 0.959^{a,b}$	$3.357 \pm 0.975^{a,b}$	6.506 ± 1.394^{b}	6.505 ± 0.775^{b}	1.433 ± 0.401^{a}
Bifidobacterium	0.008 ± 0.005^{a}	0.004 ± 0.002^{a}	0.005 ± 0.003^{a}	0.043 ± 0.024^{a}	0.540 ± 0.202^{b}
Asaccharobacter	$0.020 \pm 0.005^{a,b}$	0.030 ± 0.006^{a}	0.008 ± 0.003^{b}	0.011 ± 0.003^{b}	$0.016 \pm 0.004^{a,b}$
Mucispirillum	0.059 ± 0.018^{a}	0.057 ± 0.013^{a}	0 ± 0^{b}	0 ± 0^{b}	0.061 ± 0.012^{a}
OTUs					
OTU_21 (Ruminococcus bromii; X85099 **97%**	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0.001 ± 0.001^{a}	1.889 ± 0.547^{b}
OTU_61 (bacterium NLAE-zl-H2; JX006253) **99%**	$0.585 \pm 0.138^{a,c}$	1.046 ± 0.243^{a}	$0.516 \pm 0.219^{a,b,c}$	0.082 ± 0.047^{b}	0.399 ± 0.121^{c}
OTU_6 (Clostridium disporicum (T); DSM 5521; Y18176) **99%**	2.735 ± 0.597^{a}	4.295 ± 2.026^{a}	3.114 ± 0.635^{a}	2.734 ± 0.560^{a}	7.692 ± 0.927^{b}
OTU_52 (bacterium ASF500; ASF 500; AF157051) **91%**	0.640 ± 0.089^{a}	0.514 ± 0.070^{a}	$0.334 \pm 0.117^{a,b}$	$0.363 \pm 0.116^{a,b}$	0.221 ± 0.058^{b}
OTU_24 (Gram-negative bacterium cL10-2b-4; AY239469) **89%**	0.261 ± 0.056^{a}	0.225 ± 0.107^{a}	0.340 ± 0.157^{a}	0.250 ± 0.063^{a}	2.358 ± 0.334^{b}
OTU_92 (butyrate-producing bacterium M62/1; AY305309) **97%**	$0.297 \pm 0.099^{a,b}$	$0.304 \pm 0.053^{a,b}$	0.676 ± 0.196^{a}	$0.528 \pm 0.176^{a,b}$	0.127 ± 0.021^{b}
OTU_3 (Akkermansia muciniphila (T); Muc; AY271254) **100%**	$3.448 \pm 1.131^{a,b}$	$4.049 \pm 1.170^{a,b}$	7.929 ± 1.647^{a}	7.942 ± 0.908^{a}	1.744 ± 0.481^{b}

(A vs P p=0.07; A vs AP p=0.06 C vs P p=0.06; C vs AP p = 0.06)					
OTU_12 (Bifidobacterium choerinum; 7 VI A; JQ002525) **99%**	0.009 ± 0.006^{a}	0.005 ± 0.002^{a}	0.006 ± 0.004^{a}	0.055 ± 0.030^{a}	0.653 ± 0.242^{b}
OTU_81 (Lachnospiraceae bacterium 605; AB700364) **99%**	0.023 ± 0.010^{a}	0.025 ± 0.013^{a}	0.037 ± 0.012^{a}	0.033 ± 0.011^{a}	1.666 ± 0.285^{b}
OTU_4 (Lachnospiraceae bacterium 607; AB700365) **96%**	2.810 ± 1.637^{a}	2.040 ± 1.151^{a}	2.919 ± 0.792^{a}	3.678 ± 1.565^{a}	10.469 ± 1.731^{b}
OTU_160 (bacterium YE57; AY442821) **97%**	$0.032 \pm 0.005^{a,b}$	$0.033 \pm 0.009^{a,b}$	0.019 ± 0.005^{a}	0.023 ± 0.004^{a}	0.063 ± 0.010^{b}
OTU_253 (Turicibacter sp. LA61; AB727348) **100%**	0.014 ± 0.004^{a}	0.025 ± 0.021^{a}	0.013 ± 0.004^{a}	0.013 ± 0.006^{a}	0.094 ± 0.020^{b}
OTU_60 (Roseburia sp. 499; JX629259) **97%**	0.587 ± 0.132^{a}	0.621 ± 0.162^{a}	0.005 ± 0.004^{b}	0.008 ± 0.005^{b}	$0.296 \pm 0.110^{a,b}$
OTU_53 (Gram-negative bacterium cTPY-13; AY239461) **84%**	$0.562 \pm 0.060^{a,c}$	0.638 ± 0.085^{c}	$0.367 \pm 0.059^{a,b}$	$0.513 \pm 0.038^{a,c}$	0.266 ± 0.018^{b}
OTU_374 (Intestinimonas sp. GM5; LN876649) **90%**	0.010 ± 0.005^{a}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0.017 ± 0.004^{a}
OTU_237 (rumen bacterium R-7; AB239481) **94%**	0.025 ± 0.005^{a}	$0.027 \pm 0.008^{a,b}$	$0.019 \pm 0.004^{a,b}$	0.007 ± 0.003^{b}	$0.017 \pm 0.004^{a,b}$
OTU_642 (Clostridiales bacterium 24-4c; HQ452852) **95%**	0.262 ± 0.040^{a}	0.385 ± 0.072^{a}	0.346 ± 0.079^{a}	0.338 ± 0.073^{a}	0.097 ± 0.022^{b}
OTU_152 (Adlercreutzia equolifaciens; FJC-B20; AB306662) **99%**	0.150 ± 0.015^{a}	0.155 ± 0.015^{a}	0.080 ± 0.010^{b}	0.071 ± 0.012^{b}	0.100 ± 0.011^{b}
OTU_375 (Clostridiales bacterium CIEAF 021; AB702937) **100%**	$0.032 \pm 0.009^{a,b}$	$0.038 \pm 0.008^{a,b}$	0.023 ± 0.004^{a}	$0.047 \pm 0.010^{a,b}$	0.062 ± 0.010^{b}
OTU_142 (Aestuariispira insulae (T); AH-MY2; KF876014) **86%**	$0.071 \pm 0.030^{a,c}$	0.123 ± 0.043^{a}	0 ± 0^{b}	$0.012 \pm 0.007^{b,c}$	$0.029 \pm 0.013^{a,c}$
OTU_69 (Lachnospiraceae bacterium 19gly4; AF550610) **94%**	0.181 ± 0.069^{a}	0.070 ± 0.026^{a}	0.129 ± 0.036^{a}	0.104 ± 0.032^{a}	0.001 ± 0.001^{b}
OTU_214 (Lactobacillus murinus; ONS2; AY324630) **100%**	0.032 ± 0.015^{a}	0.019 ± 0.011^{a}	0.025 ± 0.009^{a}	0.027 ± 0.011^{a}	0.095 ± 0.020^{b}
OTU_147 (Clostridium methylpentosum (T); DSM 5476; Y18181) **91%**	$0.060 \pm 0.010^{c,b}$	$0.034 \pm 0.016^{a,c}$	$0.045 \pm 0.019^{a,c}$	0.022 ± 0.006^{a}	0.141 ± 0.026^{b}
OTU_136 (Anaerotruncus colihominis (T); 14565; AJ315980) **96%**	0.093 ± 0.017^{a}	$0.086 \pm 0.027^{a,b}$	$0.101 \pm 0.023^{a,b}$	0.175 ± 0.040^{a}	0.042 ± 0.010^{b}
OTU_162 (Lactobacillus johnsonii; GAL-2; AB295648) **100%**	$0.041 \pm 0.023^{a,b}$	0.005 ± 0.004^{a}	$0.025 \pm 0.014^{a,b}$	0.016 ± 0.012^{a}	0.067 ± 0.020^{b}

OTU_418 (Clostridium sp.; LIP5; Y12289) **96%**	0 ± 0^{a}	0 ± 0^{a}	0.002 ± 0.001^{a}	0 ± 0^{a}	0.040 ± 0.016^{b}
OTU_461 (Clostridiales bacterium CIEAF 021; AB702937) **94%**	$0.034 \pm 0.019^{a,b}$	0.002 ± 0.001^{a}	$0.037 \pm 0.031^{a,b}$	0.259 ± 0.124^{b}	$0.088 \pm 0.044^{a,b}$
OTU_157 (Lachnospiraceae bacterium 19gly4; AF550610) **99%**	0.039 ± 0.004^{a}	0.049 ± 0.008^{a}	0.021 ± 0.004^{b}	$0.042 \pm 0.012^{a,b}$	$0.037 \pm 0.007^{a,b}$
OTU_314 (Flexistipes group bacterium HRI1cae; AF059187) **100%**	0.024 ± 0.009^{a}	0.027 ± 0.008^{a}	0 ± 0^{b}	0 ± 0^{b}	0.022 ± 0.006^{a}
OTU_31 (Clostridium sp. YIT 12069; AB491207) **88%**	2.062 ± 0.502^{a}	1.661 ± 0.454^{a}	$1.080 \pm 0.344^{a,b}$	0.181 ± 0.144^{b}	$0.899 \pm 0.411^{a,b}$
OTU_200 (Clostridiales bacterium CIEAF 026; AB702939) **89%**	$0.025 \pm 0.010^{a,b}$	$0.015 \pm 0.010^{a,b}$	$0.013 \pm 0.007^{a,b}$	0 ± 0^{a}	0.059 ± 0.019^{b}
OTU_309 (Candidatus Soleaferrea massiliensis AP7; JX101688) **98%**	0.001 ± 0.001^{a}	0 ± 0^{a}	0 ± 0^{a}	0.001 ± 0.001^{a}	0.016 ± 0.006^{b}
OTU_383 (Intestinimonas sp. GD4; LN870298) **95%**	0.005 ± 0.002^{b}	$0.002 \pm 0.002^{a,b}$	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
OTU_380 (Christensenella minuta (T); YIT 12065; AB490809) **83%**	$0.003 \pm 0.002^{a,b}$	0.008 ± 0.002^{a}	0.001 ± 0.001^{b}	$0.003 \pm 0.001^{a,b}$	$0.006 \pm 0.005^{a,b}$
OTU_340 (Clostridium sp. Culture Jar-13; AB622826) **87%**	0 ± 0^{a}	0 ± 0^{a}	$0.004 \pm 0.004^{a,b}$	0.015 ± 0.005^{b}	0 ± 0^{a}
OTU_261 (Eubacterium sp. WAL 14571; FJ687606) **94%**	$0.009 \pm 0.003^{a,b}$	0.017 ± 0.004^{a}	$0.015 \pm 0.007^{a,b}$	$0.010 \pm 0.004^{a,b}$	0.002 ± 0.002^{b}
OTU_342 (Peptostreptococcaceae bacterium canine oral taxon 221; PV088; JN713384) **95%**	0.002 ± 0.001^{a}	$0.004 \pm 0.002^{a,b}$	0.002 ± 0.002^{a}	0.003 ± 0.003^{a}	0.011 ± 0.003^{b}
OTU_359 (Clostridium sp. YIT 12069; AB491207) **98%**	$0.001 \pm 0.001^{a,b}$	0.005 ± 0.002^{a}	$0.001 \pm 0.001^{a,b}$	0 ± 0^{b}	0 ± 0^{b}
OTU_595 (Lachnospiraceae bacterium 14-2; DQ789124) **98%**	0.059 ± 0.020^{a}	$0.041 \pm 0.023^{a,b}$	0.058 ± 0.024^{a}	0.073 ± 0.033^{a}	0.008 ± 0.006^{b}
OTU_241 (Ruminococcaceae bacterium GD6; LN881596) **92%**	0.010 ± 0.003^{b}	0.032 ± 0.007^{a}	$0.007 \pm 0.003^{b,c}$	0.001 ± 0.001^{c}	$0.007 \pm 0.003^{b,c}$
OTU_278 (Candidatus Soleaferrea massiliensis AP7; JX101688) **93%**	0.001 ± 0.001^{b}	$0.006 \pm 0.002^{a,b}$	0.018 ± 0.009^{a}	0.012 ± 0.005^{a}	$0.003 \pm 0.001^{a,b}$
OTU_286 (Denitrobacterium detoxificans; NPOH3; AF079506) **89%**	$0.004 \pm 0.004^{a,b}$	0.012 ± 0.012^{a}	0 ± 0^{b}	0 ± 0^{b}	0.007 ± 0.003^{a}
OTU_337 (Eubacterium plexicaudatum; ASF 492; AF157054) **94%**	0.007 ± 0.002^{b}	0 ± 0^{a}	0.001 ± 0.001^{a}	0.005 ± 0.002^{b}	$0.004 \pm 0.002^{a,b}$

OTU_882 (Lachnospiraceae bacterium G11; KC143064) **96%**	0.005 ± 0.002^{a}	0.003 ± 0.002^{a}	0.007 ± 0.005^{a}	0.007 ± 0.003^{a}	0.343 ± 0.119^{b}
OTU_338 (Candidatus Soleaferrea massiliensis AP7; JX101688) **94%**	0.003 ± 0.002^{a}	$0.005 \pm 0.003^{a,b}$	0.013 ± 0.007^{a}	$0.003 \pm 0.002^{a,b}$	0 ± 0^{b}
OTU_102 (bacterium WH2-11; JQ269302) **99%**	$0.534 \pm 0.087^{a,b}$	$0.296 \pm 0.295^{a,c}$	$0.551 \pm 0.151^{a,b,c}$	0.781 ± 0.147^{b}	0.216 ± 0.050^{c}
OTU_7 (Gram-negative bacterium cL10-2b-4; AY239469) **91%**	0.903 ± 0.903^{b}	$3.114 \pm 1.664^{a,b}$	4.735 ± 1.576^{a}	5.872 ± 1.609^{a}	5.320 ± 1.496^{a}
OTU_213 (Clostridium thermocellum; L09173) **91%**	0.002 ± 0.002^{a}	0.002 ± 0.002^{a}	$0.054 \pm 0.047^{a,b}$	0.004 ± 0.004^{a}	0.022 ± 0.006^{b}
OTU_154 (Peptostreptococcaceae bacterium canine oral taxon 019; OB001; JN713180) **86%**	0.026 ± 0.012^{a}	0.014 ± 0.006^{a}	0.037 ± 0.013^{a}	$0.050 \pm 0.020^{a,b}$	0.133 ± 0.029^{b}
OTU_66 (Roseburia intestinalis (T); L1-82; AJ312385) **93%**	$0.347 \pm 0.102^{a,b}$	$0.351 \pm 0.088^{a,b}$	$0.484 \pm 0.151^{a,b}$	0.817 ± 0.150^{b}	0.147 ± 0.059^{a}
OTU_94 (Clostridium sp. YIT 12070; AB491208) **92%**	0.206 ± 0.049^{a}	0.197 ± 0.056^{a}	0.236 ± 0.082^{a}	0.165 ± 0.048^{a}	0.031 ± 0.015^{b}
OTU_266 (Lactococcus lactis; UK1560; from a traditional fermented milk; AJ271851) **100%**	0.089 ± 0.069^{a}	0.029 ± 0.008^{a}	0.026 ± 0.007^{a}	0.032 ± 0.010^{a}	0 ± 0^{b}
OTU_294 (Clostridium sp. Culture-41; AB622820) **92%**	0.018 ± 0.009^{a}	0.034 ± 0.010^{a}	$0.014 \pm 0.011^{a,b}$	$0.002 \pm 0.001^{a,b}$	0 ± 0^{b}
OTU_582 (Lachnospiraceae bacterium 595; AB700362) **97%**	0.002 ± 0.002^{a}	0.012 ± 0.011^{a}	0.004 ± 0.002^{a}	0.042 ± 0.020^{b}	0.001 ± 0.001^{a}
OTU_409 (Clostridium methylpentosum (T); DSM 5476; Y18181) **92%**	0 ± 0^{a}	0.001 ± 0.001^{a}	0.001 ± 0.001^{a}	0 ± 0^{a}	0.018 ± 0.007^{b}
OTU_390 (Clostridiales bacterium oral taxon F32; VO026; HM099644) **95%**	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0.014 ± 0.010^{b}

3.5.5 Gut Microbiota Composition at the End of the Study Contributes to Growth Rates and Fasting Glucose Levels

Germ-free mice receiving cecal microbiota transplant from offspring (Wk17) of prebiotic consuming dams (PRE) had significantly lower weight gain on day 7, 10, 14, 17 and 21 post-FMT when compared to the ABT group (Figure 3.10A). Similarly, the ABT+PRE group displayed reduced growth rates with a stronger phenotype in the first 10 days post-FMT (Figure 3.10A, d21). The ABT group showed a trend towards increased weight gain on d17 post-FMT compared to CTR ($p=0.108$; Figure 3.10A). No differences were seen in fat mass (Figure 3.10B), lean mass (Figure 3.10C) and fasting insulin levels (Figure 3.10E) between the groups at the end of the study. The ABT group had higher fasting glucose levels compared to PRE and a trend towards an increase was seen when compared to CTR ($p=0.120$) and ABT+PRE ($p=0.176$) groups (Figure 3.10D). ABT+PRE group had higher hepatic triglyceride levels compared to PRE group only (Figure 3.10F).

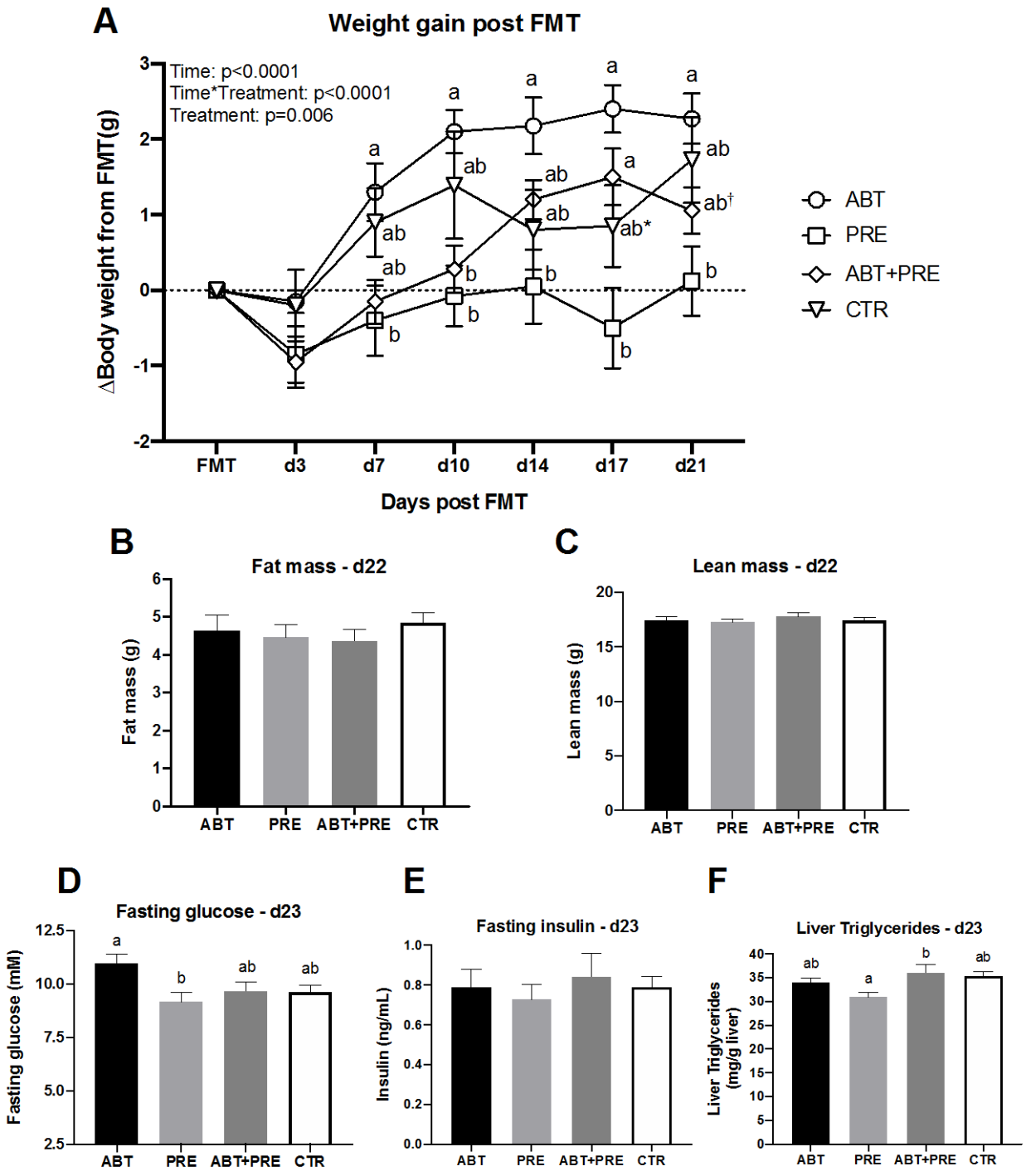


Figure 3.10 Fecal microbiota transplant (FMT) from ABT male offspring significantly increased growth rates and fasting glucose levels when compared to PRE offspring only.

(A) Growth rates post FMT, *p=0.108 between ABT and CTR, †p=0.064 between ABT and

ABT+PRE; (B) Fat mass; (C) Lean mass; (D) Fasting glucose; (E) Fasting insulin; (F) Liver triglyceride (n=8-12 mice/group). Results are shown as mean ± SEM. Labelled means without a common superscript letter differ ($p < 0.05$). ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; CTR, control.

Discussion

Several association studies in humans[199,200] and in animals[130] show increased risk of obesity in children when mothers received antibiotics during pregnancy. This is the first report showing that not only are offspring at higher risk for obesity following maternal antibiotic treatment, but the pregnant/lactating dams themselves remain heavier postpartum, with metabolic hormone impairment and increased fat accumulation. Most importantly, we demonstrate that co-administering prebiotics with antibiotics protects the mothers and prevents obesity risk in their offspring, with accompanying changes in the gut microbiota.

Maternal antibiotic administration during pregnancy/lactation impaired maternal weight loss following birth, increased their fat mass and hepatic triglycerides, disrupted their metabolic hormones and changed their microbial profiles. The disruption to maternal microbial profiles at birth, with high levels of *Enterobacteriaceae* and low levels of penicillin-sensitive *Lactobacillus*[130,133] could partly explain impaired maternal postpartum weight loss. Shifts in gut microbiota composition occur during normal pregnancy with third trimester microbiota showing promotion of adiposity and insulin resistance[211]. Therefore, since our antibiotic exposure began in the third trimester and was continued throughout lactation, altered third trimester microbiota in dams treated with antibiotics could interfere with the typical microbial changes that occur during lactation, thereby delaying expected weight loss after birth. In a healthy pregnancy, higher levels of Proteobacteria (i.e. *Enterobacteriaceae*) are seen in the third trimester[211]. However, our study showed an increase in *Enterobacteriaceae* at birth in antibiotic-treated dams only, which was associated with elevated fat mass and leptin levels at the end of lactation, both of which are expected to occur naturally in the third trimester of pregnancy[211].

Importantly, maternal prebiotic co-administration with antibiotics prevented the obese phenotype and adverse antibiotic-associated outcomes in dams. Examination of the

longitudinal changes in microbial profiles in prebiotic groups provides insight into the leaner phenotype of PRE and ABT+PRE dams. We observed normalized *Enterobacteriaceae* and *Lactobacillus* levels at birth in the dams and a gradual increase in *Bifidobacterium* spp. and *Collinsella* levels throughout lactation when prebiotics were consumed, both known to be associated with a lean phenotype[212]. Indeed, our correlation analysis confirmed a negative association between *Collinsella aerofaciens* and body weight of dams. Our findings are in line with a recent study in rats where maternal prebiotic intake increased the abundance of health promoting gut bacteria *Bifidobacterium* spp., decreased energy intake and prevented hepatic steatosis in their offspring[213]. Higher levels of *Bifidobacterium* spp.[188] in other studies have been associated with improvements in hepatic steatosis, reductions in fat mass and improvements in glycemia[196,214,215], findings also observed in our study.

Beyond compositional changes in gut microbiota driven by prebiotics, increased levels of the satiety hormone GLP-1 may explain reduced caloric intake alongside lower body weight, fat mass and liver triglycerides in PRE and ABT+PRE groups, findings also reported in human[190] and animal[205] studies. Interestingly, several gut microbial OTUs correlated with GLP-1 levels in our study. Another potential benefit of increased secretion of the incretin GLP-1 in prebiotic dams was improved glycemic control, a mechanism described previously[216]. From our previous work involving maternal serum metabolomics analysis[202], markers of increased insulin sensitivity and decreased fatty acid import were found in dams supplemented with oligofructose further explaining the lower body weight/body fat/liver triglycerides seen in prebiotic-treated dams. The metabolic changes occurring in dams, driven by antibiotic and prebiotic-induced microbial changes, markedly impacted the risk of obesity in the offspring.

We confirm the increased risk of obesity in offspring of antibiotic-treated dams shown previously in mice[130]. Male and female ABT offspring had accelerated early-life growth rates and were prone to obesity upon HFS metabolic challenge; a finding that could be due in part to the aforementioned maternal microbiota disruption. Higher abundance of *Clostridium coccoides* with increased capacity to harvest energy from diet[50] was seen in both sexes of offspring at weaning and this increase could explain accelerated weight gain of the offspring especially as no differences in food intake were observed.

Since most of the early colonizers are derived from initial exposures with maternal body sites[51], a mother is probably the most influential source for the colonization of the infant's gut microbiota (contact during birth, nursing, and early feeding)[45,51]. While it is possible that there was minor indirect exposure of the offspring to the antibiotics via maternal licking/grooming of the pups, it is very likely that ABT offspring were colonized with dysbiotic maternal microbiota at birth; high levels of pro-inflammatory *Enterobacteriaceae* and low levels of *Lactobacillus*. Previous experiments in GF mice demonstrated that microbiota transplant from donor mice treated with LDP to recipient GF mice accelerated weight gain and fat mass[130]. Furthermore, the gut microbiota of recipient mice in days 1-14 post transfer was characterized by reduced *Lactobacillus* that was already detected prior to phenotype development[130]. This finding demonstrated that loss of *Lactobacillus* during a critical developmental window can be detrimental later in life[130]. Specifically, a negative correlation between *Lactobacillus* and total/fat mass and a positive correlation with expression of transcription factors and cytokines important for T helper immune cells differentiation and function was reported[130]. At weaning, we observed lower *Lactobacillus* in male offspring only, partially explaining why males had more negative outcomes (increased adiposity and liver triglycerides) upon maternal antibiotic exposure than females. In line with this, a trend towards higher fasting insulin levels and insulin resistance was seen in male offspring only and this phenotype could be explained by higher levels of *Clostridium* cluster XI at the end of the study. A previous report in mice showed that greater abundance of bile acid-modifying bacteria *Clostridium* cluster XI was associated with diabetes[217], possibly because of the production of proinflammatory bile acids such as taurodeoxycholic acid (TDCA) and deoxycholic acid (DCA)[218]. The stronger phenotype we observed in male versus female offspring is in line with animal and human studies investigating maternal antibiotic exposure during pregnancy[20,130,141,155,165].

Interestingly, ABT females had higher levels of *Enterobacteriaceae* at weaning compared to all other groups, which might be due to the spike in *Enterobacteriaceae* in ABT dams at birth. The reason for sex differences in microbiota composition at weaning (low *Lactobacillus* in males and high *Enterobacteriaceae* in females) and end of the study (high *Clostridium* cluster XI in males only) might be due to the involvement of sex

hormones in microbiota development[219]. Experiments in mice have shown that the microbiota of females and castrated males were more similar than when microbiota of females were compared to males[219]. In addition, sex hormones also influence the production of bile acids, which are known to influence gut microbiota[220].

The negative outcomes of maternal antibiotic exposure are in line with other animal studies[130,204], but we are the first to show that prebiotics can prevent undesirable metabolic and microbial outcomes. Male and female prebiotic offspring (PRE, ABT+PRE) had higher abundances of *Bifidobacterium* spp. and *Collinsella* at weaning, which could explain their lower body weight throughout the study. Infants who acquired higher levels of *Bifidobacterium* spp. and *Collinsella* early in life had lower adiposity at 18 months of age[212]. Prebiotic oligofructose is known to have a "bifidogenic" effect[188], which is crucial given bifidobacteria role in early gut and immune system development and promotion of a lean phenotype[213]. Here we show that maternal prebiotic co-administration with antibiotics rescues many of the negative outcomes in offspring with males benefiting more than females, which could be due to the greater metabolic dysfunction seen in males. Similar sex-specific observations have been reported in humans with men having a greater cardiovascular risk, impaired glucose homeostasis, dyslipidemia, increased inflammatory markers and a higher prevalence of metabolic syndrome, despite having similar body mass indexes (BMIs) as females[221].

Since the impact of direct LDP exposure on growth promotion was investigated previously with FMT experiments[130], we decided to investigate whether indirect LDP exposure also contributes to the weight gain seen post-HFS metabolic challenge in our male offspring. In order to determine whether gut microbiota contributed to increased body weights/fat mass/isulin at the end of the study, FMT with cecal matter from male pups was transferred into GF mice. While trends were observed for increased growth rates in ABT-microbiota recipient mice, PRE-microbiota recipient mice displayed significantly lower weight gain throughout the study demonstrating the importance of gut microbiota to the lean phenotype in these rats. While we could not transfer the increased adiposity/hepatic triglyceride ABT phenotype to GF mice, it is possible this occurred because microbiota recipients were not challenged with a HFS diet, which is necessary to reveal obesity risk.

Our data show that maternal antibiotic/prebiotic intake during pregnancy/lactation had the greatest impact on gut microbiota composition in offspring immediately after weaning with differences dissipating over time, particularly after consumption of a HFS diet, when offspring for the most part had converged to a similar microbiota profile. This is not surprising, as we know that diet is the major driver of gut microbiota composition[222] and even though microbiota of the offspring had almost recovered by the end of the study, the phenotype persisted due to disruptions that were present during a critical developmental window as explained previously[130]. To fully understand host-microbiota interactions influenced by prebiotic/antibiotic administration, further studies, including fecal microbiota transplant earlier in life (e.g. when pups are still suckling on the ABT-treated dams or shortly after weaning) as well as characterizing metabolic differences in the offspring even before weaning, are needed.

3.6 Conclusions

In this study we show that antibiotics administered during pregnancy/lactation diminish postpartum weight loss in dams and increase obesity risk in offspring. Furthermore, antibiotic administration to dams increased their fat mass and hepatic triglycerides, disrupted their metabolic hormones and changed microbial profiles throughout lactation. For the first time we demonstrate the protective role of maternal co-administration of prebiotics with antibiotics. Accelerated weight gain of the antibiotic offspring and increased fat mass and hepatic triglycerides (male offspring only) were prevented with maternal prebiotic co-administration. Should this finding translate to humans, it could represent a non-invasive means of reducing the metabolic risks to offspring that is associated with maternal antibiotic use during pregnancy and lactation.

3.7 Acknowledgements

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agency had no role in the design of the study or preparation of this manuscript, and had no influence on the data collection, analysis and interpretation or manuscript publication. The oligofructose was provided by Beneo (Mannheim, Germany).

3.8 Author Contributions

Teja Klancic and Raylene A. Reimer conceived the experiments. Teja Klancic, Ashley Choo, Jodi E. Nettleton, Faye Chleilat, and Nicole A. Cho carried out the experiments. Isabelle Laforest-Lapointe, Marie-Claire Arrieta, Alissa C. Nicolucci and Teja Klancic performed gut microbiota bioinformatics. Teja Klancic performed all other data analysis. Teja Klancic drafted the paper and all authors critically revised the manuscript for important intellectual content. Raylene A. Reimer received funding and had primary responsibility as the study supervisor.

CHAPTER FOUR: CONCURRENT PREBIOTIC INTAKE REVERSES INSULIN RESISTANCE INDUCED BY EARLY-LIFE PULSED ANTIBIOTIC IN RATS

4.1 Abstract

Pulsed antibiotic exposure (PAT) early in life increases risk of insulin resistance and obesity. Prebiotics improve metabolic health and reduce fat mass. Our aim was to examine if co-administering prebiotic with PAT reduces obesity risk in rat pups weaned onto a high fat/sucrose (HFS) diet. Sprague-Dawley rats were mated and their pups cross-fostered at 19 days old. Dams and their litters were randomized to: 1) control [CTR], 2) antibiotic [ABT] (azithromycin), 3) prebiotic [PRE] (10% oligofructose (OFS)), 4) antibiotic+prebiotic [ABT+PRE] and 5) lean control [LEAN]. Three pulses of antibiotics/prebiotics were administered at d19-21, d28-30 and d37-39 of life and animals euthanized when 10 weeks old. At d21, rats were weaned onto a HFS with prebiotic groups receiving 10% OFS in their diet until d39. Male and female rats given antibiotics (ABT) had higher body weight than any other group at the end of the study. The PAT phenotype was stronger in ABT males than females, where increased fat mass, hyperinsulinemia and insulin resistance were present and all reversible with prebiotics. Reduced hypothalamic/hepatic expression of insulin receptor substrates was seen in males only, explaining their greater insulin resistance. In females, insulin resistance was improved with prebiotics and normalized to lean control. ABT reduced *Lactobacillaceae* and increased *Bacteroidaceae* in both sexes. Therapeutic doses of antibiotic administered to rats mirrored the concentration commonly used in children for an acute infection. Antibiotics increased body weight, impaired insulin production and insulin sensitivity, but the effects were reversed with prebiotic co-administration in a sex-specific manner.

4.2 Introduction

Gut microbiota has co-evolved with its human host, conferring a wide-range of metabolic, nutritional, and immunological benefits for the human host[49]. However, not all host-microbiota interactions are beneficial and in the case of dysbiosis, disruptions to the microbial community can contribute to obesity and other metabolic diseases[50]. Alterations to the gut microbiota are more likely to occur early in life due to microbial instability and higher likelihood of perturbations by external factors such as antibiotics[55]. Antibiotics are the most commonly prescribed therapeutic agents around the world[131]. Exposure to antibiotics in early life is of particular concern given that approximately 90% of exclusively breast-fed infant microbiota are bifidobacteria, which are highly susceptible to antibiotics[148]. Reduced bifidobacteria abundance is found in individuals with higher BMI[98,99] and a negative correlation has been shown between *Bifidobacterium* and visceral adiposity[6]. Disrupting gut microbiota during critical developmental windows can have lifetime consequences due to limited "microbial pressure" resulting in abnormal immune system maturation[71,72]. In addition to disrupting gut microbiota development, antibiotics also impair the ability of microbes to deal with stressors such as a high fat, high sugar Western diet[147].

Early experiments in farm animals showed that low dose (subtherapeutic) administration of antibiotic promotes growth. However, the mechanisms by which this stimulation occurred were unknown[140]. Later experiments using early-life sub-therapeutic antibiotic treatment (STAT) in mice[130] demonstrated altered microbiota, increased body weight/fat mass and worsened metabolic outcomes after STAT. Fecal microbiota transplant showed that the obese phenotype could be transferred from donor to germ free recipient mice demonstrating the important causative role the microbial community plays in early-life antibiotic-induced obesity risk[130]. Importantly, we recently demonstrated that the negative metabolic and phenotypic side effects of STAT were prevented when co-administering prebiotic oligofructose with antibiotic in pregnant and lactating dams and their offspring (Klancic et al. personal communication).

The most recent definition of a prebiotic is "a substrate that is selectively utilized by host microorganisms conferring a health benefit"[188]. Prebiotics, particularly chicory root-derived inulin and oligofructose have been shown in multiple studies in humans and

animals to reduce body weight, fat mass, increase serum satiety hormones, reduce inflammation and increase the levels of health promoting *Bifidobacterium*[25,188,206,213].

Given the high rates of antibiotic use, particularly in children, there is a need to identify strategies that will attenuate the known risks associated with their exposure in early life. Therefore, our objective was to mimic paediatric antibiotic use and determine whether the negative metabolic outcomes of early life antibiotic use could be mitigated by co-administering prebiotic oligofructose. To mimic human antibiotic treatment, we administered therapeutic doses of azithromycin, a commonly used antibiotic in humans[223] and animals[224]. In a 3-year cohort study involving more than 30 million individuals in the USA, azithromycin was found to be the most commonly prescribed antibiotic between 2013 and 2015[223]. Importantly, our schedule of administration (10mg/kg/day for 3 consecutive days) mimicked administration regimen used in children[225]. Key parameters that we investigated were body weight and composition, metabolic hormones (insulin/leptin/GLP-1), longitudinal microbiota changes and hypothalamic/hepatic gene expression involved in insulin signalling such as insulin receptor substrate (IRS)-1 and IRS-2[226].

Our results show increased body weight of males and females given antibiotics early in life with greater fat mass accumulation in males. In addition, antibiotics impaired insulin sensitivity, hepatic and hypothalamic gene expression as well as microbiota development (reduced *Lactobacillaceae* and increased *Bacteroidaceae* levels). To our knowledge, we are the first to demonstrate that prebiotic co-administration to the therapeutic doses of antibiotics prevents the negative metabolic side effects of antibiotics.

4.3 Materials and methods

4.3.1 Animals and diets

A total of 30 Sprague Dawley rats (10wk old, n=20 females, n=10 males) were obtained from Charles River Laboratories (Saint Constant, QC, Canada). Two animals from the same dam were housed together on a 12 h light–dark cycle in a temperature (20–22°C) and humidity (41-60%)-controlled room. After 2 weeks of acclimatization, rats were mated to generate pups for inclusion in the intervention study. Within 24 hours of birth, litters were culled to 10 pups (n=5 males, n=5 females). Pregnant/lactating dams

consumed normal chow (Lab Diet 5001, United States) throughout the study. Pups were cross-fostered at 19 days of age (to reduce litter effect) and dams with their newly composed litters were randomized to (n=20 pups/group): 1) control [CTR], 2) antibiotic [ABT] (azithromycin; dose 10 mg/kg/day; oral suspension concentration of 200 mg/5ml; Zithromax; Pfizer[227]), 3) prebiotic [PRE] (10% oligofructose (OFS) oral suspension/diet, 10% wt/wt, Orafti P95, Beneo-Orafti Inc.), 4) antibiotic+prebiotic [ABT+PRE] and 5) lean conrol [LEAN]. The first pulse of antibiotics/prebiotics was administered before weaning from d19-21 of life through a feeding dropper. The second and third pulse of antibiotic were given d28-30 and d37-39, respectively. Animals were weaned onto a high-fat/high sucrose diet (HFS) at d21 (diet #102412; Dyets, Bethlehem, PA,USA), with prebiotic groups (P and AP) containing 10% OFS in their diet. Prebiotic groups remained on the diet until the last pulse of antibiotics. A fifth lean reference group (n=20) was maintained on control chow diet throughout the study. Animals were euthanized for tissue collection at two time points: wk7 – after the last antibiotic pulse and wk10 – end of study (n=10 pups/group per time point). The 10% OFS dose was selected based on previous experiments showing reductions in fat mass[201] and increase in *Bifidobacterium spp*. and *Lactobacillus spp.* favoring a lean phenotype[89,203]. The amount of azithromycin given to the pups was calculated based on their body weight. This dose (10 mg/kg/day, 3 consecutive days, 3 pulses) and type of antibiotic is therapeutic for rodents and mirrors the concentration commonly used for human children for an acute infection[228]. Ethical and study protocol approval was granted by the University Of Calgary Animal Care Committee (Protocol #: AC15-0079).

4.3.2 Tissue and blood collection

Following a 12-hour overnight fast, animals were euthanized with over-anesthetization with isoflurane and aortic cut. Tissues (cecum, liver and hypothalamus) from the rats at 7 and 10 weeks of age were weighed, collected and snap frozen in liquid nitrogen. All tissues were stored in -80°C until analysis. Fasted blood samples were collected at 7 and 10 weeks of age for determination of glucose, insulin and leptin according to our previous work[205].

4.3.3 Insulin tolerance test (ITT)

An insulin tolerance test was performed in week 9 of life (n=10 rats/group). After a 5-hour fast, rats received an insulin load (0.75U/kg) through an intraperitoneal injection. Blood glucose levels during the ITT were measured at 0 (baseline), 15, 30, 60, 90 and 120 minutes. Blood glucose was determined immediately at each time point using OneTouch Verio Blood Glucose Meter (OneTouch®). Insulin resistance was assessed using Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) using the formula: (fasting insulin concentration*fasting glucose concentration)/22.5, a method used previously[229] and validated in rat model[230].

4.3.4 Food and fluid intake

Water and food intake were measured throughout the study at 4 different time points (week 5 of life – during their second antibiotic pulse; week 6 of life – during the third antibiotic pulse; week 8 of life – before insulin tolerance test and week 10 of life – end of the study). Since two animals were housed together, the average daily food consumption per animal was calculated by dividing cage consumption by two to acquire food intake/animal/day.

4.3.5 Body weight and composition

Body weight was measured weekly throughout the study. At week 7 and week 10 of life, animals were lightly anaesthetized with isoflurane and body composition was measured via dual energy x-ray absorptiometry (DXA) scan with software for small animals (Hologic ODR 4500; Hologic).

4.3.6 Serum LPS

Blood was collected via tail bleed at week 7 and portal vein at week 10 of life. Blood was centrifuged at 1200×g for 10 minutes and serum stored at -80°C until analysis. For the analysis, samples were heated for 1 hour at 70°C and LPS measured using PyroGene Recombinant Factor C Endotoxin Detection Assay (Lonza) as described previously.[195,213]

4.3.7 Real-time PCR analysis

Hypothalamic and liver samples were processed for real-time PCR as previously described[231]. Briefly, total RNA was extracted using TRIzol reagent (Invitrogen,

Carlsbad, CA) and reverse transcription to cDNA was performed using 2µg of total RNA and cDNA synthesis kit (Invitrogen, Carlsbad, CA). Primers for liver-related genes (IRS-1, IRS-2, FAS, LPL and housekeeping: GAPDH) and primers for hypothalamus-related genes (POMC, AGRP, NPY, IL-10, IRS-1, IRS-2 and housekeeping: β-actin) are listed in Table 4.1. The mRNA levels were calculated using the $2^{-\Delta CT}$ method[213].

Table 4.1 Primer sequences for RT-qPCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Liver and Hypothalamus		
FAS	GCCGTGGTGCTGGAGATTG	TGCCGAGGTTGGTGAGGAAG
LPL	CCCTACAAAGTATTCCATTACC	CCGTGTAAATCAAGAAGGAG
IRS-1	CTCTGCTTCTGCTTCTGTTAC	TGGTTATGGTTGGGACTTAGG
IRS-2	AAGATAGCGGGTACATGCGAAT	GCAGCTTAGGGTCTGGGTTCT
IL-10	AGTCAGCCAGACCCACATG	GCAACCCAAGTAACCCTTAAAG
POMC	AGGTTAAGGAGCAGTGACTAAG	AGCAGAATCTCGGCATCTTC
AGRP	CGTGCTACTGCCGCTTCTTC	CCCTGCCTTTGCCCAACATC
NPY	GCTCGTGTGTTTGGGCATTC	GAGATTGATGTAGTGTCGCAGAG
β-Actin	ATCAAGATCATTGCTCCTCCTG	GACTCATCGTACTCCTGCTTG
GAPDH	CAAGTTCAACGGCACAGTCAAG	ACATACTCAGCACCAGCATCAC

4.3.8 Fecal collection and 16S rRNA Illumina sequencing

Fecal samples were collected repeatedly throughout the study: after the first (day 22 of life), second (day 31 of life), and third (day 40 of life) antibiotic pulse and at the end of study (beginning of week 10/day 64 of life). Using ≈ 250mg of fecal matter, total bacterial DNA was extracted using a FastDNA Spin Kit for feces (MP Biomedicals). Fecal DNA was quantified (PicoGreen kit, Invitrogen) and diluted to 20 ng/µl for sequencing. Microbial sequencing was performed on the MiSeq Illumina platform at the Centre for Health Genomics and Informatics (University of Calgary). The V3 and V4 regions of the 16S rRNA gene were amplified and the protocol involved a two-step, tailed PCR approach that generated ready-to-pool amplicon libraries as described previously[206]. The pooled and indexed library set was denatured, diluted, and sequenced in paired-end modus on an Illumina MiSeq (Illumina Inc., San Diego, USA). Sequences were checked for quality, trimmed, merged, and checked for chimeras using the DADA2[232] and phyloseq[207] packages for R (R Development Core Team; http://www.R-

project.org). A bacterial community matrix was built from the resulting unique Amplicon Sequence Variants (ASV). To reduce biases introduced by DNA amplification (i.e. PCR) and sequencing errors, we excluded any ASV that was found less than five times in the community matrix. This resulted in a final dataset of 4,336,788 quality sequences and 2280 ASVs. The number of sequences per sample varied from 2732 to 20,533, with a mean of 12,945.64.

4.3.9 Statistical analysis (16S rRNA Illumina sequencing)

To account for potential heteroskedasticity in community beta-diversity dispersion and avoid the loss of information through rarefaction[209], we performed a variance stabilizing transformation[209,210] prior to any statistical tests. Changes in gut bacterial community structure (beta-diversity) were assessed statistically using Permutational Multivariate ANalysis Of Variance (PERMANOVA) and visualized using Principal Component Analysis (PCoA) based on Bray-Curtis dissimilarities. To explore further the changes in taxonomical community structure, we tested for significant changes in relative abundance of the 15 most dominant bacterial families using non-parametric Kruskal-Wallis tests followed by post-hoc Dunn tests with Benjamin-Holmes False Discovery Rate (FDR) correction. To estimate gut bacterial alpha-diversity, we measured the Chao1 (richness) and Shannon indices. We used an ANOVA on a linear mixed-model to test for significant differences in alpha-diversity between treatments and time points, followed by a Tukey's post-hoc test. Subject ID was implemented as a random factor to account for repeated measures in our dataset. All data is presented as mean ± standard error of the mean (SEM).

4.3.10 Statistical analysis – biological and qPCR outcomes

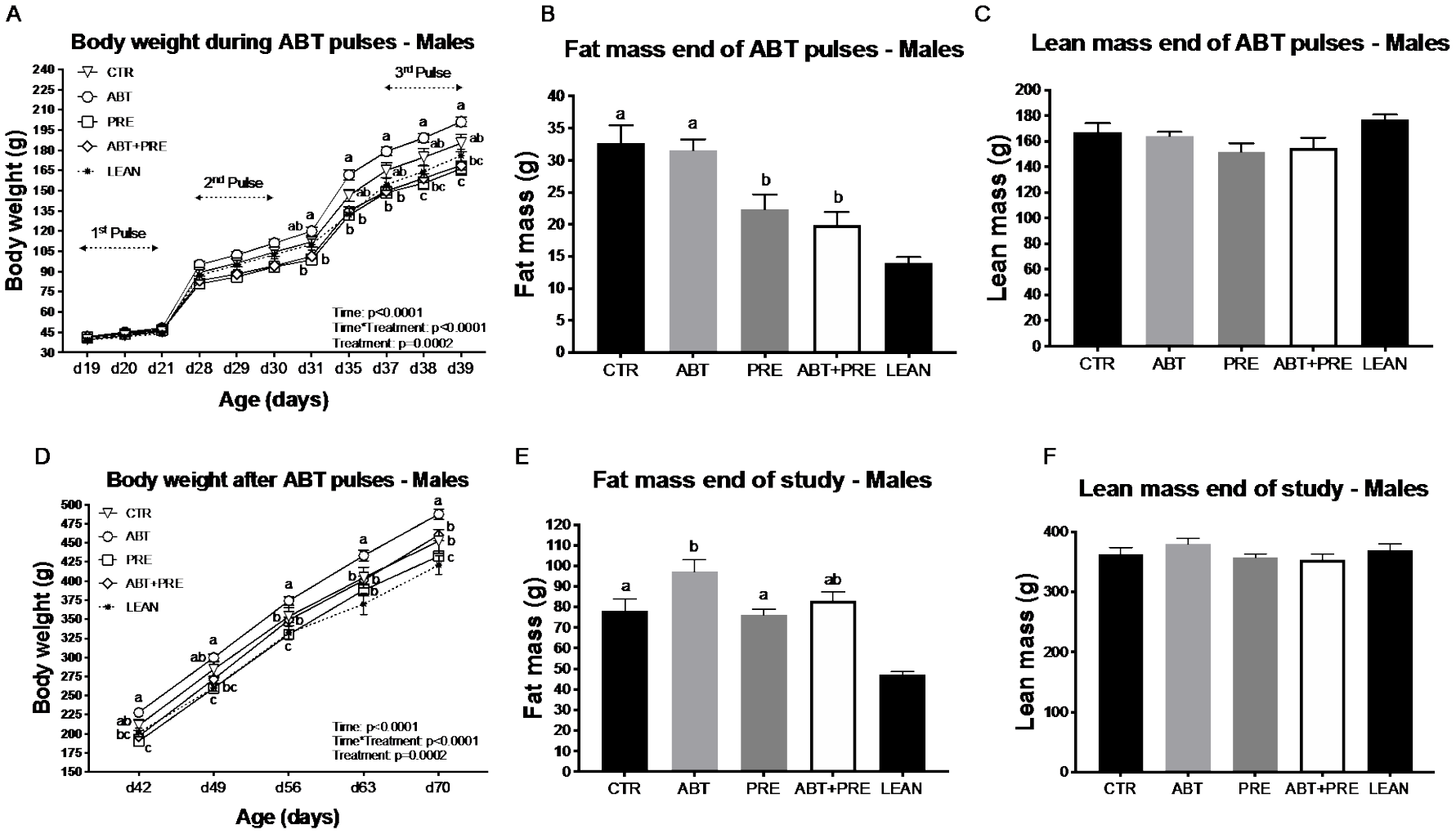
All data is presented as mean ± standard error of the mean (SEM). Boxplots were made to identify outliers and normality was assessed using the Shapiro-Wilk test. If the data was normally distributed ($p>0.05$), parametric one-way analysis of variance (ANOVA) with Tukey's post-hoc tests was used. For longitudinal and timed data (body weights, ITT), statistical tests on univariate response variable were performed using a linear mixed-model for repeated measures, followed by an ANOVA with Tukey's post hoc when appropriate. The lean reference group was not included into statistical analysis. In

all tests, significance was set at $p<0.05$. Statistical analyses and graphs were made using Prism version 7.0d (GraphPad Software, La Jolla, CA, USA).

4.4 Results

4.4.1 Pulsed early life antibiotic exposure increases body weight and leads to insulin resistance

Previous animal studies demonstrated increased body weight after sub-therapeutic antibiotic exposure[130,204], therefore we aimed to investigate whether therapeutic doses of azithromycin resulted in a similar phenotype. Male rats given antibiotics alone (ABT group) became ~20% heavier than the prebiotic groups (PRE, ABT+PRE) by the end of the second antibiotic pulse (d31, Figure 4.1A) and remained heavier until the end of the pulses (Figure 4.1A). The differences in body weight between ABT and prebiotic groups were due to increased fat mass (Figure 4.1B) and not lean mass (Figure 4.1C). In addition, prebiotic groups had lower leptin levels (Figure 4.1G), fasting glucose (Figure 4.1H), fasting insulin (Figure 4.1I) and were more insulin sensitive (Figure 4.1J) when compared to ABT and CTR at the end of antibiotic pulses. There was a trend toward insulin resistance in the ABT group (Figure 4.1J; ABT vs CTR, $p=0.077$). Once the antibiotics/prebiotics were discontinued and the experimental groups continued on the HFS diet, the ABT group became heavier than all other groups (Figure 4.1D) despite no differences in caloric intakes between the groups (Figure 4.1R). The increase in body weight in the ABT group was due to increased fat mass (Figure 4.1E) and not lean mass (Figure 4.1F). Interestingly, the male antibiotic groups (ABT, ABT+PRE) had increased leptin levels compared to CTR at the end of the study (Figure 4.1K), however, fasting insulin levels (Figure 4.1L) as well as insulin resistance (Figure 4.1M) was strictly increased in the ABT group. The insulin tolerance test confirmed these results as 15 minutes after the insulin load, higher glucose levels were seen in ABT group compared to prebiotic groups indicating insulin resistance (Figure 4.1N). Higher LPS levels were seen in the ABT group at week 10 compared to CTR and PRE groups (Figure 4.2A), but not at week 7 (Figure 4.2B). While the animals were on a prebiotic diet, cecum mass was increased in the prebiotic groups (Figure 4.1O) and remained slightly elevated until the end of study (Figure 4.1P).


A
Body weight during ABT pulses - Males
CTR
ABT
PRE
ABT+PRE
LEAN
1st Pulse
2nd Pulse
3rd Pulse
Body weight (g)
Age (days)
d19 d20 d21 d28 d29 d30 d31 d35 d37 d38 d39
Time: p<0.0001
Time*Treatment: p<0.0001
Treatment: p=0.0002
B
Fat mass end of ABT pulses - Males
Fat mass (g)
CTR ABT PRE ABT+PRE LEAN
C
Lean mass end of ABT pulses - Males
Lean mass (g)
CTR ABT PRE ABT+PRE LEAN
D
Body weight after ABT pulses - Males
CTR
ABT
PRE
ABT+PRE
LEAN
Body weight (g)
Age (days)
d42 d49 d56 d63 d70
Time: p<0.0001
Time*Treatment: p<0.0001
Treatment: p=0.0002
E
Fat mass end of study - Males
Fat mass (g)
CTR ABT PRE ABT+PRE LEAN
F
Lean mass end of study - Males
Lean mass (g)
CTR ABT PRE ABT+PRE LEAN

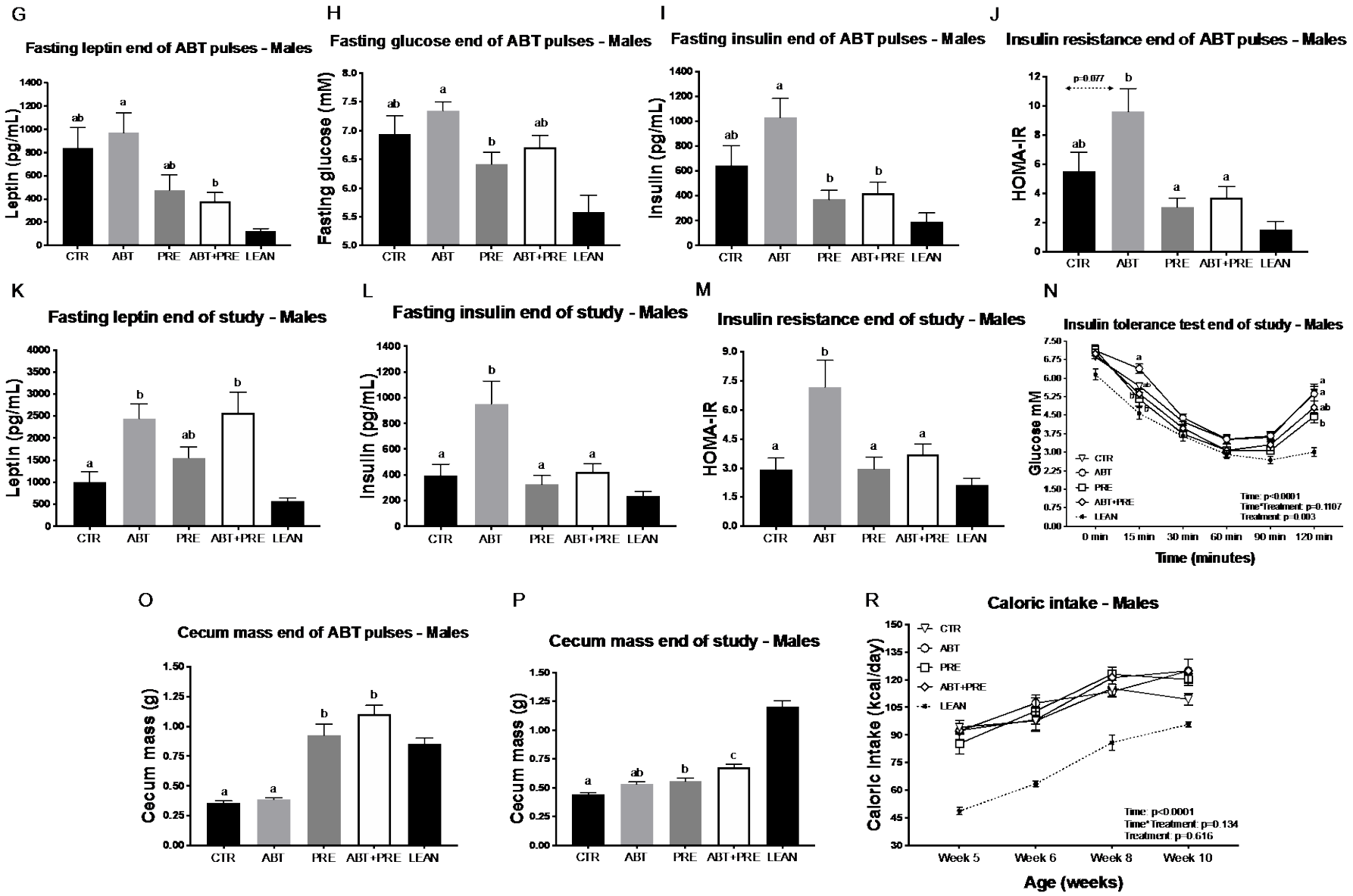

G
Fasting leptin end of ABT pulses - Males
Leptin (pg/mL)
CTR ABT PRE ABT+PRE LEAN
H
Fasting glucose end of ABT pulses - Males
Fasting glucose (mM)
CTR ABT PRE ABT+PRE LEAN
I
Fasting insulin end of ABT pulses - Males
Insulin (pg/mL)
CTR ABT PRE ABT+PRE LEAN
J
Insulin resistance end of ABT pulses - Males
HOMA-IR
p=0.077
CTR ABT PRE ABT+PRE LEAN
K
Fasting leptin end of study - Males
Leptin (pg/mL)
CTR ABT PRE ABT+PRE LEAN
L
Fasting insulin end of study - Males
Insulin (pg/mL)
CTR ABT PRE ABT+PRE LEAN
M
Insulin resistance end of study - Males
HOMA-IR
CTR ABT PRE ABT+PRE LEAN
N
Insulin tolerance test end of study - Males
Glucose mM
CTR
ABT
PRE
ABT+PRE
LEAN
Time: p<0.0001
Time*Treatment: p=0.1107
Treatment: p=0.003
0 min 15 min 30 min 60 min 90 min 120 min
Time (minutes)
O
Cecum mass end of ABT pulses - Males
Cecum mass (g)
CTR ABT PRE ABT+PRE LEAN
P
Cecum mass end of study - Males
Cecum mass (g)
CTR ABT PRE ABT+PRE LEAN
R
Caloric intake - Males
Caloric Intake (kcal/day)
CTR
ABT
PRE
ABT+PRE
LEAN
Time: p<0.0001
Time*Treatment: p=0.134
Treatment: p=0.616
Week 5 Week 6 Week 8 Week 10
Age (weeks)

Figure 4.1 Pulsed early life antibiotic exposure increases body weight, fat mass and insulin levels/resistance in males, all reversible with prebiotic co-administration

(A) Body weight of males during antibiotic pulses (n=8 rats/group). (B) Fat mass and (C) Lean mass of males at the end of antibiotic pulses (n=7-10 rats/group). (D) Body weight of males after antibiotic pulses (n=8 rats/group). (E) Fat mass and (F) Lean mass of males at the end of the study (n=7-10 rats/group). Portal vein leptin (G) and insulin (I) levels of males measured at the end of antibiotic pulses (n=7-10 rats/group). (H) Fasting glucose of males measured end of antibiotic pulses via tail bleed (n=8-10 rats/group). (F) Insulin resistance of males at the end of antibiotic pulses (n=8-9 rats/group). Portal vein leptin (K) and insulin (L) levels of males at the end of the study (n=7-9 rats/group). (M) Insulin resistance of males at the end of the study (n=8 rats/group). (N) Glucose response to insulin in males measured by ITT at the end of the study (n=8-10 rats/group). (O and P) Cecum mass in males at the end of antibiotic pulses (O) and end of study (P) (n=7-10 rats/group). (R) Average caloric intake (kilocalories) of males calculated as the average of energy intake over 4 days measured at 4 different weeks of life (n=6-8 rats/group). Results are shown as mean ± SEM. Different superscript letters denote significant differences between the groups, $p < 0.05$. CTR, control; ATB, antibiotic; PRE, prebiotic; ATB+PRE, antibiotic+prebiotic; LEAN, lean control; d, day of life.

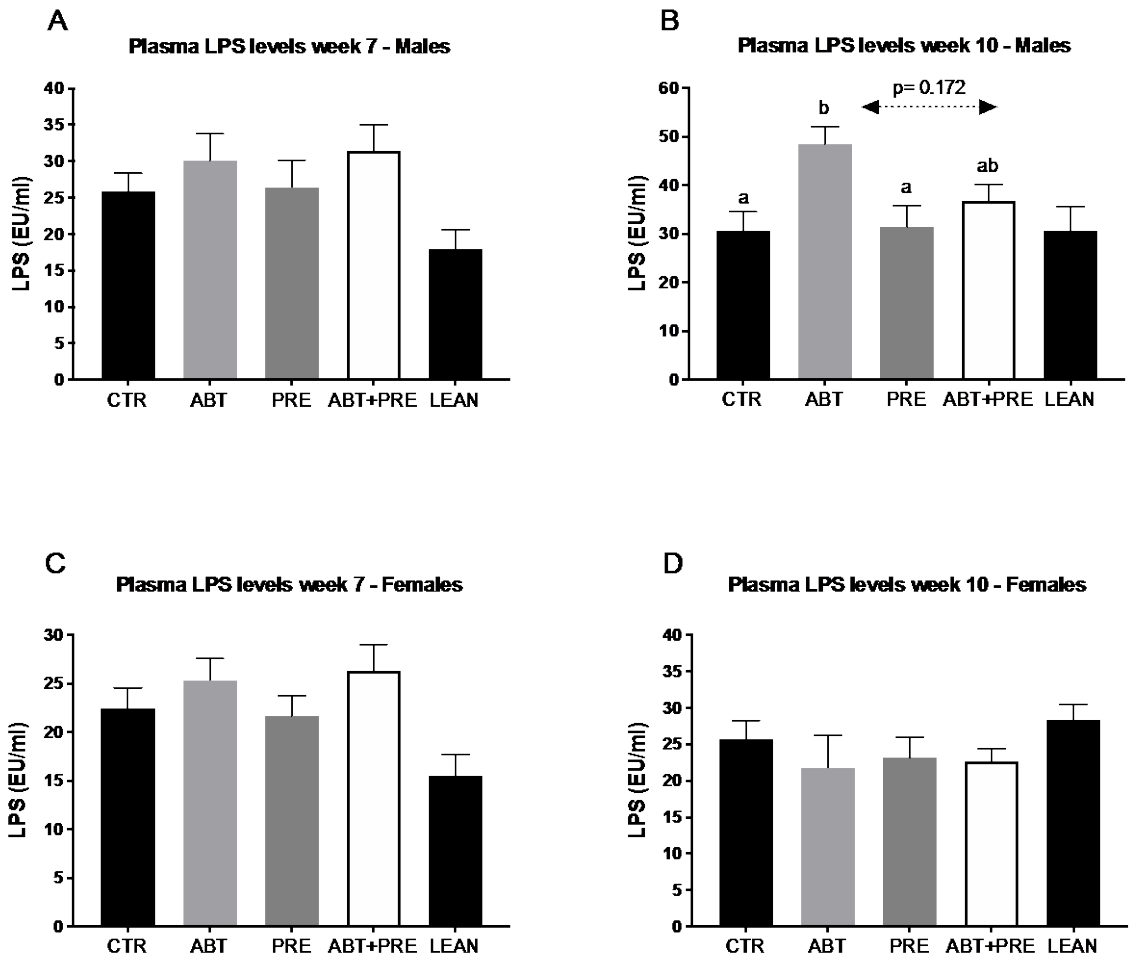
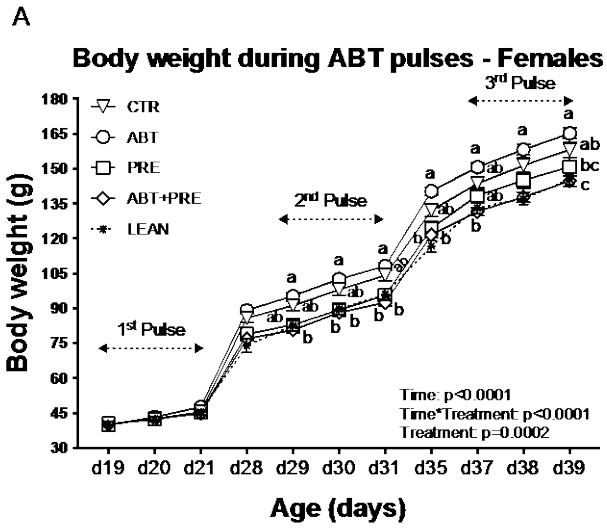


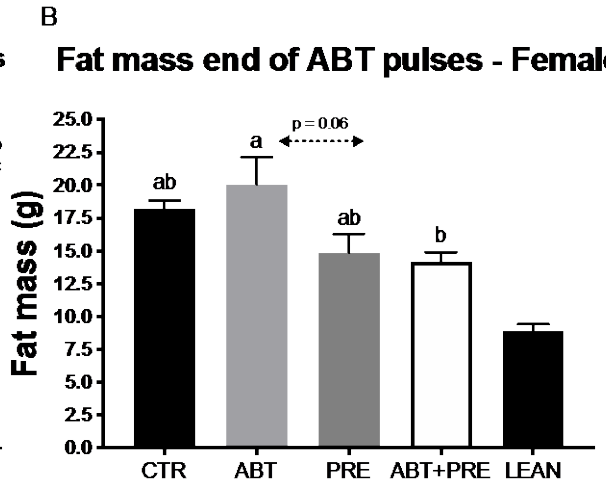
Figure 4.2 ABT male offspring display increased endotoxemia at the end of the study

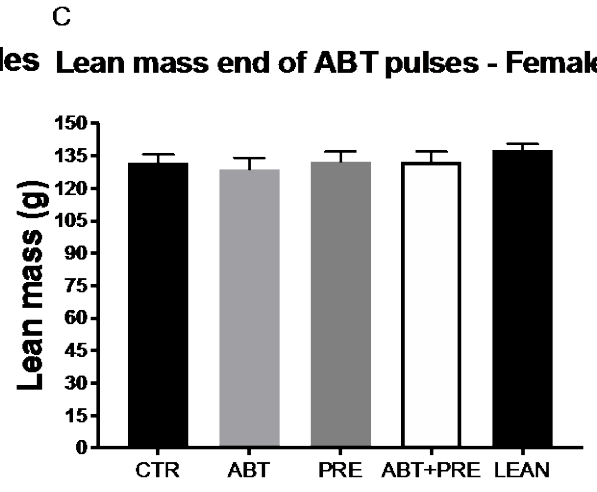
(A and B) Circulating LPS levels in males at (A) week 7 of life and (B) end of the study (n=8-10 rats per group/time point). (C and D) Circulating LPS levels in females at (C) week 7 of life and (D) end of the study (n=8-10 rats per group/time point). Different superscript letters denote significant differences between the groups, $p < 0.05$. CTR, control; ATB, antibiotic; PRE, prebiotic; ATB+PRE, antibiotic+prebiotic; LEAN, lean control.

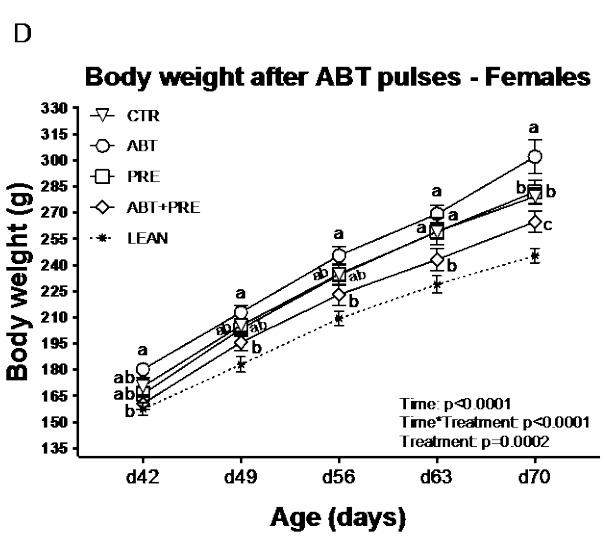
Similar to males, the female ABT group became ≈15% heavier during the second ABT pulse (d30, Figure 4.3A) and remained heavier until the end of the ABT pulses compared to the prebiotic groups (PRE, ABT+PRE). The differences in body weight were due to lower fat mass in the prebiotic groups (Figure 4.3B) and not lean mass

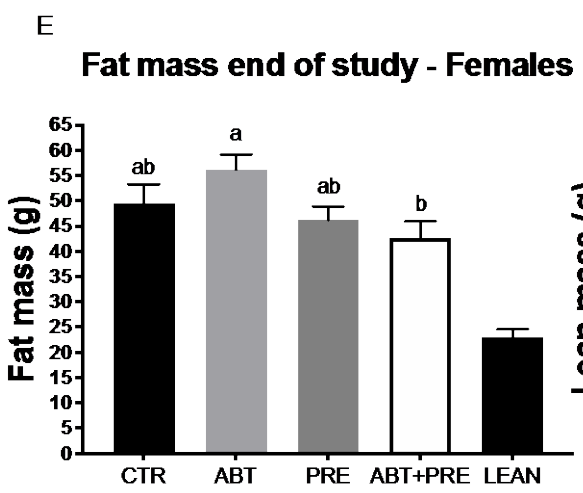
(Figure 4.3C). In addition, the prebiotic groups displayed low leptin levels (Figure 4.3G), fasting glucose (Figure 4.3H), fasting insulin (Figure 4.3I) and were more insulin sensitive (Figure 4.3J) when compared to CTR and ABT group. Their metabolic profiles at the end of the ABT pulses (Figure 4.3G-J) matched lean control levels even though they were consuming a HFS diet. At the end of the study, the ABT group was heavier compared to all other groups (Figure 4.3D) despite no differences seen in their caloric intakes (Figure 4.3R). There was a trend toward an increase in fat mass in the ABT group (Figure 4.3E) but no difference in lean mass (Figure 4.3F). A trend towards a decrease in leptin was seen in the female ABT+PRE group compared to ABT group (Figure 4.3K) and a reduction in fasting insulin (Figure 4.3L) and insulin resistance (Figure 4.3M) was observed in prebiotic groups compared to the ABT group. At the end of the ITT test, insulin resistance was seen in the ABT group compared to all other groups (Figure 4.3N). No differences in LPS levels between the groups were detected at any time point (Figure 4.2C-D). Prebiotic diet increased cecum size in the prebiotic groups at the end of the pulses (Figure 4.3O) and cecum mass remained heavier at the end of the study (Figure 4.3P).

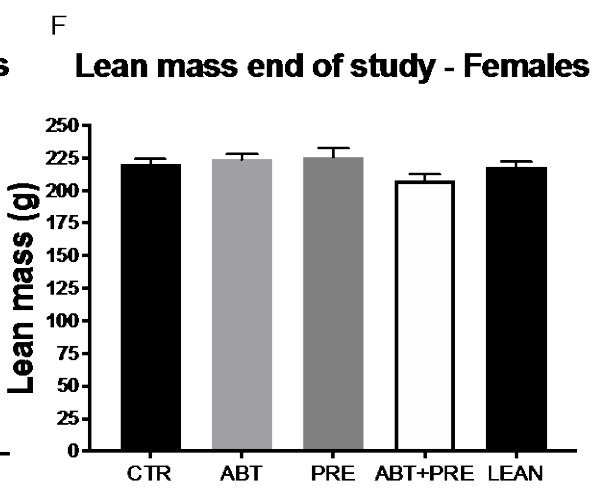

A
Body weight during ABT pulses - Females
CTR
ABT
PRE
ABT+PRE
LEAN
1st Pulse
2nd Pulse
3rd Pulse
Time: p<0.0001
Time*Treatment: p<0.0001
Treatment: p=0.0002
Body weight (g)
Age (days)
d19 d20 d21 d28 d29 d30 d31 d35 d37 d38 d39


B
Fat mass end of ABT pulses - Females
p = 0.06
Fat mass (g)
CTR ABT PRE ABT+PRE LEAN


C
Lean mass end of ABT pulses - Females
Lean mass (g)
CTR ABT PRE ABT+PRE LEAN


D
Body weight after ABT pulses - Females
CTR
ABT
PRE
ABT+PRE
LEAN
Time: p<0.0001
Time*Treatment: p<0.0001
Treatment: p=0.0002
Body weight (g)
Age (days)
d42 d49 d56 d63 d70


E
Fat mass end of study - Females
Fat mass (g)
CTR ABT PRE ABT+PRE LEAN


F
Lean mass end of study - Females
Lean mass (g)
CTR ABT PRE ABT+PRE LEAN

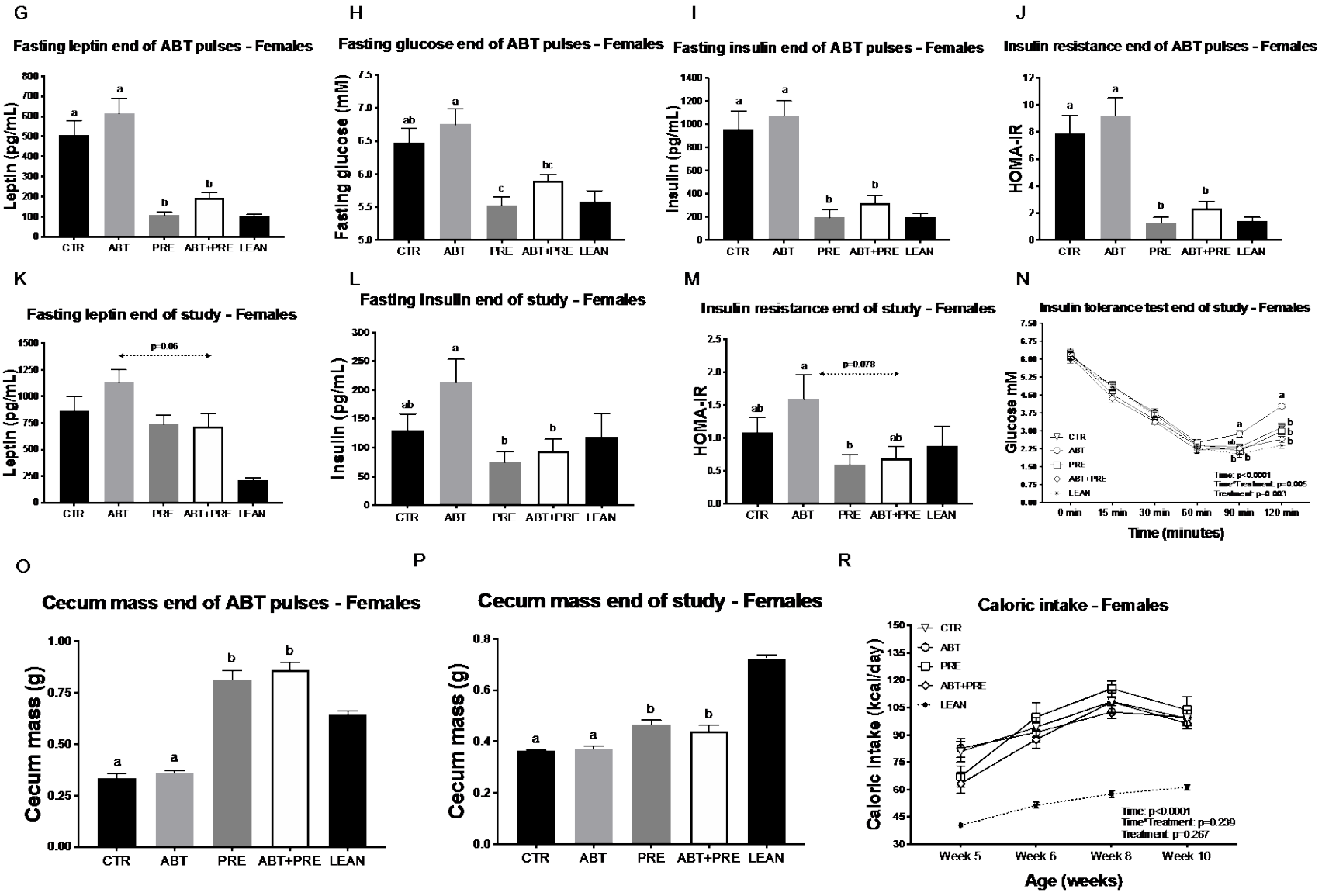

G
Fasting leptin end of ABT pulses - Females
Leptin (pg/mL)
CTR ABT PRE ABT+PRE LEAN
H
Fasting glucose end of ABT pulses - Females
Fasting glucose (mM)
CTR ABT PRE ABT+PRE LEAN
I
Fasting insulin end of ABT pulses - Females
Insulin (pg/mL)
CTR ABT PRE ABT+PRE LEAN
J
Insulin resistance end of ABT pulses - Females
HOMA-IR
CTR ABT PRE ABT+PRE LEAN
K
Fasting leptin end of study - Females
p=0.06
Leptin (pg/mL)
CTR ABT PRE ABT+PRE LEAN
L
Fasting insulin end of study - Females
Insulin (pg/mL)
CTR ABT PRE ABT+PRE LEAN
M
Insulin resistance end of study - Females
p=0.078
HOMA-IR
CTR ABT PRE ABT+PRE LEAN
N
Insulin tolerance test end of study - Females
Glucose mM
CTR
ABT
PRE
ABT+PRE
LEAN
Time: p<0.0001
Time*Treatment: p=0.005
Treatment: p=0.003
0 min 15 min 30 min 60 min 90 min 120 min
Time (minutes)
O
Cecum mass end of ABT pulses - Females
Cecum mass (g)
CTR ABT PRE ABT+PRE LEAN
P
Cecum mass end of study - Females
Cecum mass (g)
CTR ABT PRE ABT+PRE LEAN
R
Caloric intake - Females
Caloric Intake (kcal/day)
CTR
ABT
PRE
ABT+PRE
LEAN
Time: p<0.0001
Time*Treatment: p=0.239
Treatment: p=0.267
Week 5 Week 6 Week 8 Week 10
Age (weeks)

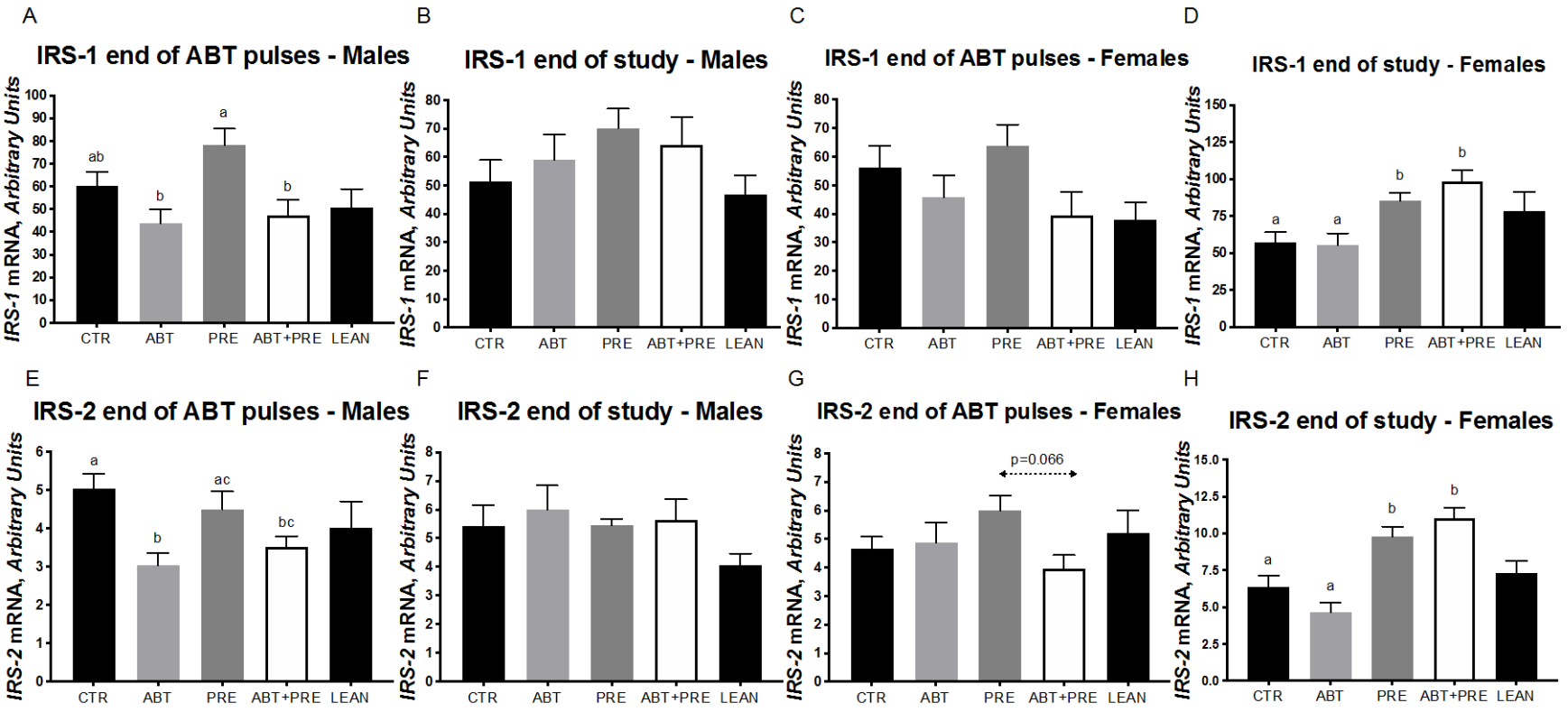
Figure 4.3 Pulsed early life antibiotic exposure increases body weight and insulin levels/resistance in females, all reversible with prebiotic co-administration

(A) Body weight of females during antibiotic pulses (n=9 rats/group). (B) Fat mass and (C) Lean mass of females at the end of antibiotic pulses (n=7-10 rats/group). (D) Body weight of females after antibiotic pulses (n=9 rats/group). (E) Fat mass and (F) Lean mass of females at the end of the study (n=8-10 rats/group). Portal vein leptin (G) and insulin (I) levels of females measured at the end of antibiotic pulses (n=8-10 rats/group). (H) Fasting glucose of females measured at the end of antibiotic pulses via tail bleed (n=8-9 rats/group). (F) Insulin resistance of females was calculated at the end of antibiotic pulses (n=8-10 rats/group). Portal vein leptin (K) and insulin (L) levels of females measured at the end of the study (n=7-9 rats/group). (M) Insulin resistance of females was calculated end of study (n=8-10 rats/group). (N) Glucose response to insulin in females measured by ITT at the end of the study (n=7-10 rats/group). (O and P) Cecum mass in females at the end of antibiotic pulses (O) and end of study (P) (n=8-10 rats/group). (R) Average caloric intake (kilocalories) of females calculated as the average of energy intake over 4 days measured at 4 different weeks of life (n=6-8 rats/group). Results are shown as mean ± SEM. Different superscript letters denote significant differences between the groups, $p < 0.05$. CTR, control; ATB, antibiotic; PRE, prebiotic; ATB+PRE, antibiotic+prebiotic; LEAN, lean control; d, day of life.

4.4.2 Early life pulsed antibiotic exposure impacts hepatic and hypothalamic gene expression

Insulin resistance was the strongest metabolic phenotype identified in this study and we therefore explored potential mechanisms that could account for this outcome. Hepatic and hypothalamic gene expression was examined at two time points (end of antibiotic pulses and end of study). In males, lower hepatic expression of the insulin receptor substrate-1 (IRS-1) and IRS-2 was seen in the ABT group at the end of the ABT pulses compared to the PRE group, but the differences were no longer present at the end of the study (Figure 4.4B; Figure 4.4F). In females, IRS-1 and IRS-2 expression was higher in prebiotic groups at the end of the study (Figure 4.4D) and the end of antibiotic pulses (Figure 4.4G), respectively. No differences were found in hepatic expression of the fatty acid synthase (FAS) gene in males at any time point (Figure 4.4I-J) or lipoprotein lipase (LPL) expression at the end of antibiotic pulses (Figure 4.4M). Lower hepatic expression of LPL was seen in ABT+PRE males at the end of the study compared to the CTR group only (Figure 4.4N). Similarly, ABT+PRE female rats had lower expression of FAS compared to CTR at the end of the pulses (Figure 4.4K). In females, higher expression of LPL was seen at the end of the study in the PRE group compared to the ABT and CTR group (Figure 4.4P). Lastly, no differences were seen in IRS-1 expression (Figure 4.4C) or LPL expression (Figure 4.4O) in females at the end of the pulses or in IRS-2 (Figure 4.4H) and FAS (Figure 4.4L) at the end of study.

Hepatic Gene Expression



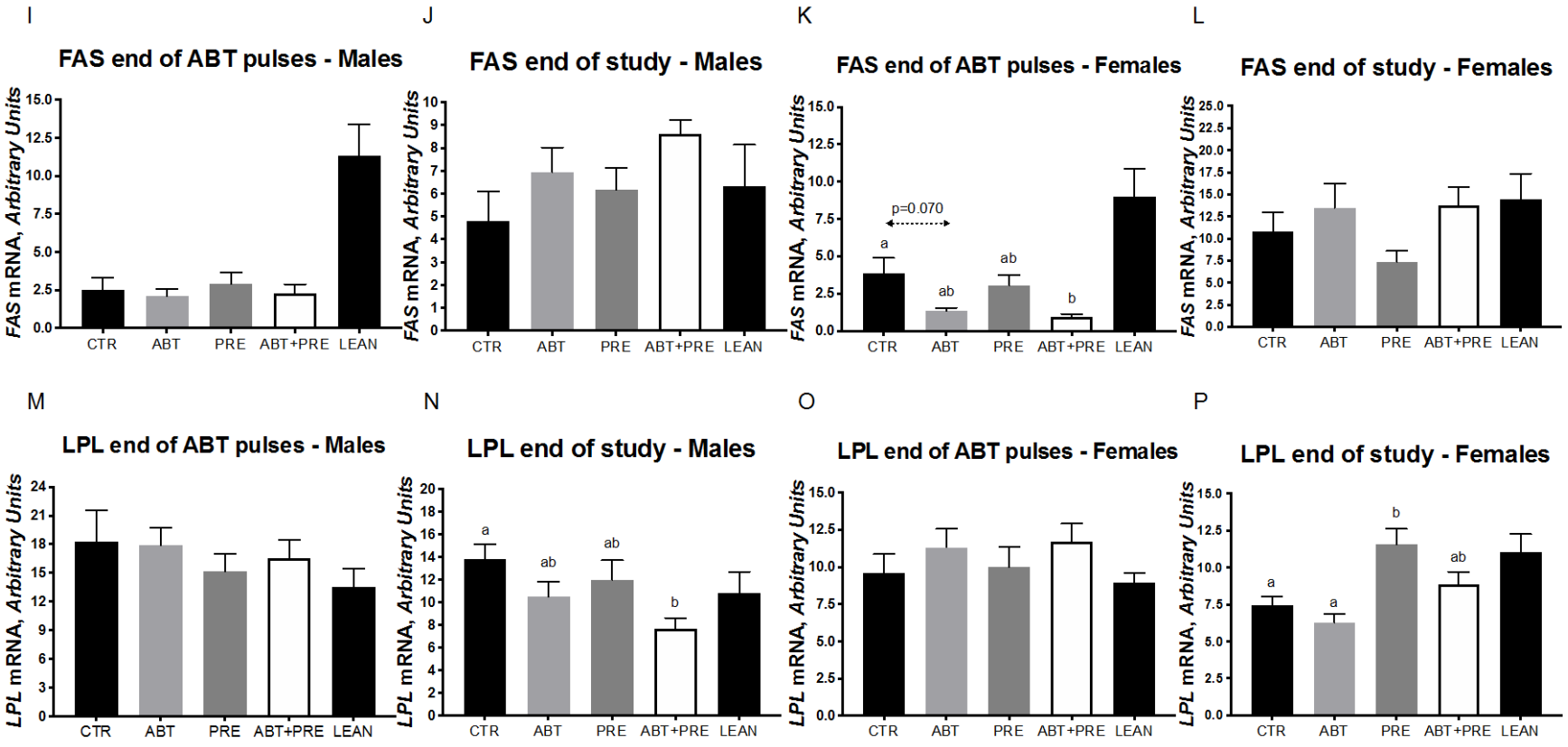
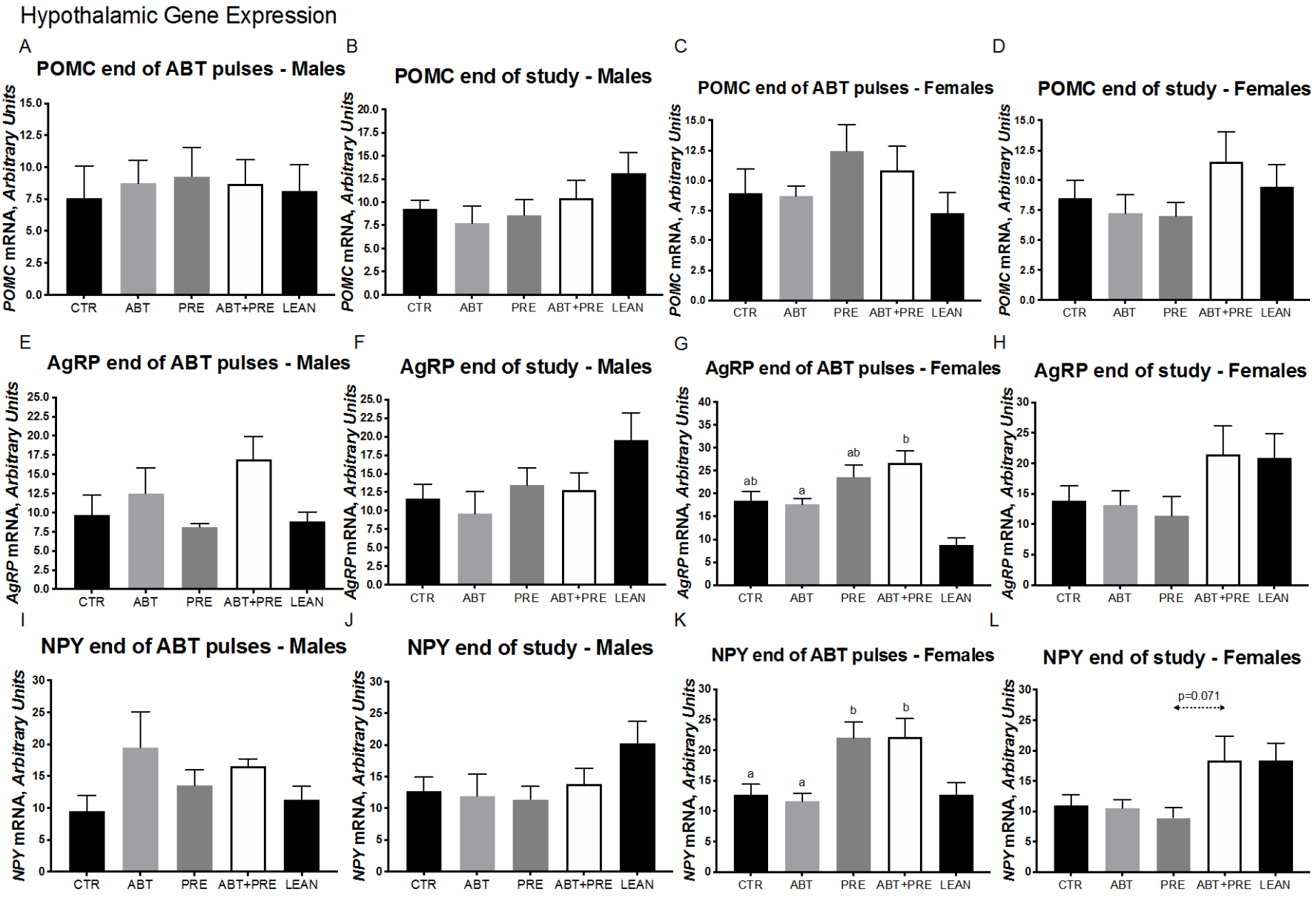


Figure 4.4 Early life pulsed antibiotic exposure impacts hepatic gene expression in males and females.

Hepatic IRS-1 expression in males (A, B) and females (C, D) after antibiotic pulses and at the end of the study respectively. Hepatic IRS-2 expression in males (E, F) and females (G, H) after antibiotic pulses and at the end of the study respectively. Hepatic FAS expression in males (I, J) and females (K, L) after antibiotic pulses and at the end of the study respectively. Hepatic LPL expression in males (M, N) and females (O, P) after antibiotic pulses and at the end of the study respectively. Results are shown as mean ± SEM

(n=7-10 rats/group). Different superscript letters denote significant differences between the groups, $p < 0.05$. CTR, control; ATB, antibiotic; PRE, prebiotic; ATB+PRE, antibiotic+prebiotic; LEAN, lean control; IRS-1, insulin receptor substrate-1; IRS-2, insulin receptor substrate-2; FAS, fatty acid synthase; LPL, lipoprotein lipase.

In the hypothalamus, lower expression of the anti-inflammatory cytokine interleukin-10 (IL-10) (Figure 4.5N), IRS-1 (Figure 4.5R) and IRS-2 (Figure 4.5V) was seen in ABT males compared to ABT+PRE males at the end of the study. Similarly, lower hypothalamic expression of IRS-1 (Figure 4.5S) was seen in the female ABT group at the end of the pulses compared to the ABT+PRE group but not at the end of the study (Figure 4.5T). While no differences in the expression of pro-opiomelanocortin (POMC) (Figure 4.5A-B), agouti-related peptide (AGRP) (Figure 4.5E-F) and neuropeptide Y (NPY) (Figure 4.5I-J) were seen in males at any time point, female prebiotic groups (PRE, ABT+PRE) had increased expression of AGRP (Figure 4.5G) and NPY (Figure 4.5K) at the end of the pulses. No differences were seen in POMC (Figure 4.5C-D), IL-10 (Figure 4.5O-P) and IRS-2 (Figure 4.5W-X) expression in females at any time point and differences in AGRP (Figure 4.5H) and NPY (Figure 4.5L) expression were diminished at the end of the study. Likewise, no differences between groups were seen at the end of the pulses for IL-10 (Figure 4.5M), IRS-1 (Figure 4.5Q) and IRS-2 (Figure 4.5U) expression in males.


Hypothalamic Gene Expression
A
POMC end of ABT pulses - Males
POMC mRNA, Arbitrary Units
CTR ABT PRE ABT+PRE LEAN
B
POMC end of study - Males
POMC mRNA, Arbitrary Units
CTR ABT PRE ABT+PRE LEAN
C
POMC end of ABT pulses - Females
POMC mRNA, Arbitrary Units
CTR ABT PRE ABT+PRE LEAN
D
POMC end of study - Females
POMC mRNA, Arbitrary Units
CTR ABT PRE ABT+PRE LEAN
E
AgRP end of ABT pulses - Males
AgRP mRNA, Arbitrary Units
CTR ABT PRE ABT+PRE LEAN
F
AgRP end of study - Males
AgRP mRNA, Arbitrary Units
CTR ABT PRE ABT+PRE LEAN
G
AgRP end of ABT pulses - Females
AgRP mRNA, Arbitrary Units
ab a ab b
CTR ABT PRE ABT+PRE LEAN
H
AgRP end of study - Females
AgRP mRNA, Arbitrary Units
CTR ABT PRE ABT+PRE LEAN
I
NPY end of ABT pulses - Males
NPY mRNA, Arbitrary Units
CTR ABT PRE ABT+PRE LEAN
J
NPY end of study - Males
NPY mRNA, Arbitrary Units
CTR ABT PRE ABT+PRE LEAN
K
NPY end of ABT pulses - Females
NPY mRNA, Arbitrary Units
a a b b
CTR ABT PRE ABT+PRE LEAN
L
NPY end of study - Females
p=0.071
NPY mRNA, Arbitrary Units
CTR ABT PRE ABT+PRE LEAN

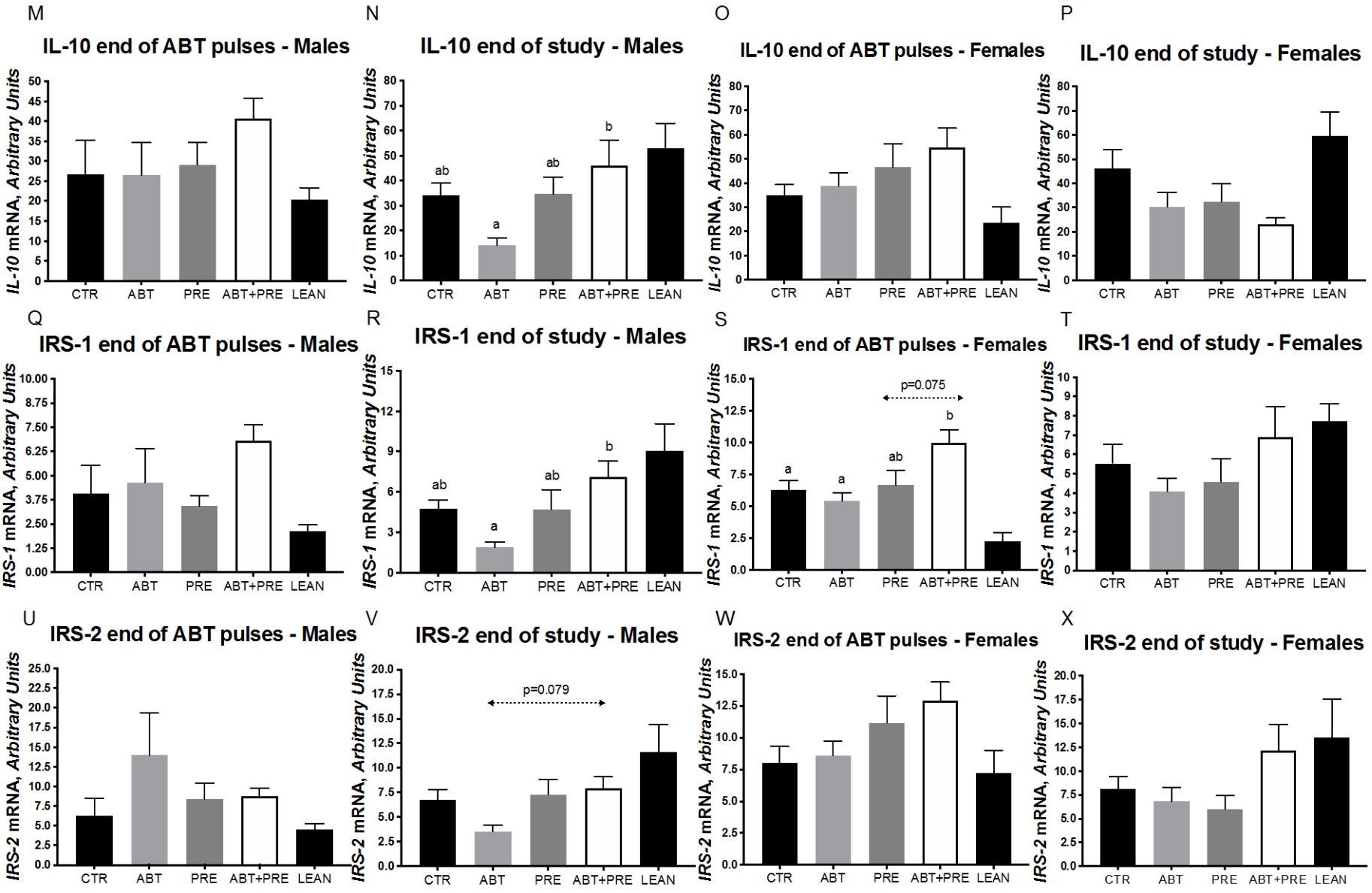

M
IL-10 end of ABT pulses - Males
IL-10 mRNA, Arbitrary Units
CTR ABT PRE ABT+PRE LEAN
N
IL-10 end of study - Males
ab a ab b
O
IL-10 end of ABT pulses - Females
P
IL-10 end of study - Females
Q
IRS-1 end of ABT pulses - Males
IRS-1 mRNA, Arbitrary Units
R
IRS-1 end of study - Males
S
IRS-1 end of ABT pulses - Females
p=0.075
T
IRS-1 end of study - Females
U
IRS-2 end of ABT pulses - Males
IRS-2 mRNA, Arbitrary Units
V
IRS-2 end of study - Males
p=0.079
W
IRS-2 end of ABT pulses - Females
X
IRS-2 end of study - Females

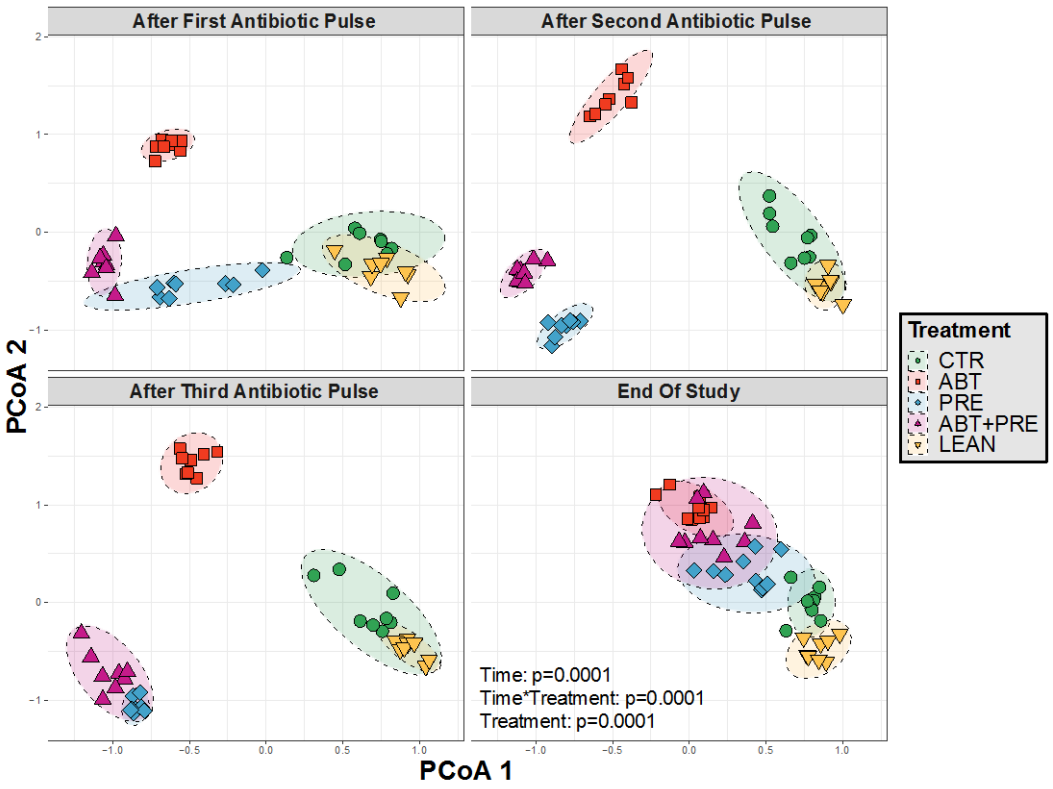
Figure 4.5 Early life pulsed antibiotic exposure impacts hypothalamic gene expression in males and females.

Hypothalamic POMC expression in males (A, B) and females (C, D) after antibiotic pulses and at the end of the study, respectively. Hypothalamic AGRP expression in males (E, F) and females (G, H) after antibiotic pulses and at the end of the study, respectively. Hypothalamic NPY expression in males (I, J) and females (K, L) after antibiotic pulses and at the end of the study, respectively. Hypothalamic IL-10 expression in males (M, N) and females (O, P) after antibiotic pulses and at the end of the study, respectively. Hypothalamic IRS-1 expression in males (Q, R) and females (S, T) after antibiotic pulses and at the end of the study, respectively. Hypothalamic IRS-2 expression in males (U, V) and females (W, X) after antibiotic pulses and at the end of the study, respectively. Results are shown as mean ± SEM (n=7-10 rats/group). Different superscript letters denote significant differences between the groups, $p < 0.05$. CTR, control; ATB, antibiotic; PRE, prebiotic; ATB+PRE, antibiotic+prebiotic; LEAN, lean control. POMC, proopiomelanocortin; AGRP, agouti-related peptide; NPY, neuropeptide Y; IL-10, interleukin-10; IRS-1, insulin receptor substrate-1; IRS-2, insulin receptor substrate-2.

4.4.3 Treatment is the main driver of gut bacterial community structure in males and females

Many environmental and lifestyle factors such as diet and antibiotics can profoundly alter gut microbiota throughout life[172]. We therefore collected fecal samples after each PAT and monitored changes over time. PERMANOVA analysis showed that treatment was the strongest driver of bacterial community structure explaining 35.5% in males and 33.8% in females (β diversity, p=0.001), whereas the experimental time point explained 12.2% of the variation in males (p=0.001; Figure 4.6A) and 13.2% in females (p=0.001; Figure 4.6B). The response in community structure in males and females changed throughout time depending on the treatment (R^2 = 18.3% for males; R^2 = 19.4%, for females; p=0.001) and the model explained a total of 66% (males) and 66.4% (females) of the changes in the bacterial community assembly. To further explore variation in gut bacterial composition, relative abundance of the 15 most dominant bacterial families were analyzed per time point and per treatment in males (Figure 4.7C) and females (Figure 4.8C). While control groups (CTR and LEAN) and the prebiotic alone group (PRE) were defined by higher levels of *Lactobacillaceae* after the antibiotic pulses in males (Figure 4.7C; Table 4.2) and females (Figure 4.8C, Table 4.3), the antibiotic group (ABT) was dominated by *Bacteroidaceae* in both sexes. Co-administration of prebiotics to antibiotics (ABT+PRE) rescued low *Lactobacillaceae* levels and reduced high *Bacteroidaceae* levels, but only after the third antibiotic pulse in males (Figure 4.7C; Table 4.2) and females (Figure 4.8C, Table 4.3). Nevertheless, prebiotics (ABT+PRE group) did not increase levels of *Porphyromonadaceae*, *Erysipelotrichaceae*, *Peptostreptococcaceae*, *Clostridiaceae,* which were depleted in ABT males (Figure 4.7C; Table 4.2) and females (Figure 4.8C; Table 4.3) after the first antibiotic pulse. Once the antibiotic treatment and prebiotic supplementation were discontinued and fecal samples were analyzed at the end of the study, most of the bacterial differences between groups disappeared in males and females, however, higher *Bacteroidaceae* levels persisted in antibiotic groups (ABT and ABT+PRE) compared to others. A reduction in alpha-diversity in males (Figure 4.7A and 4.7B) and females (Figure 4.8A and 4.8B) was seen in antibiotic/prebiotic groups when compared to controls, however, differences between groups disappeared once antibiotics/prebiotics were discontinued at the end of the study.

A (MALES)



B (FEMALES)

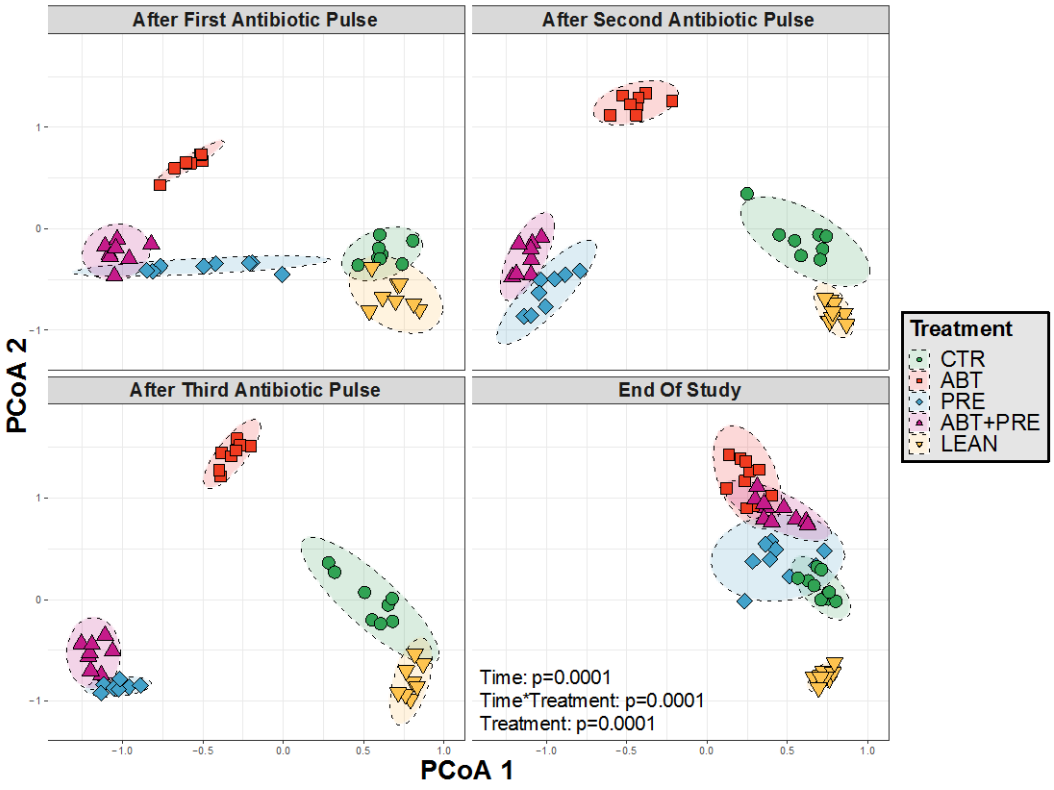


Figure 4.6 Between-group variations in beta-diversity of gut bacterial communities in male and female rats at different time points: after the first, second, third antibiotic exposure, and at the end of the study.

(A) Principal component analysis (PCoA) ordination of variation in beta-diversity of gut bacterial communities based on Bray-Curtis dissimilarities among male (A) and female (B) rats (n=8-10 rats per group/time point). Statistical significance of the effect of treatment and experimental time points on gut bacterial community structure was tested with a Permutational Analysis of Variance (PERMANOVA). CTR, control; ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; LEAN, lean control.

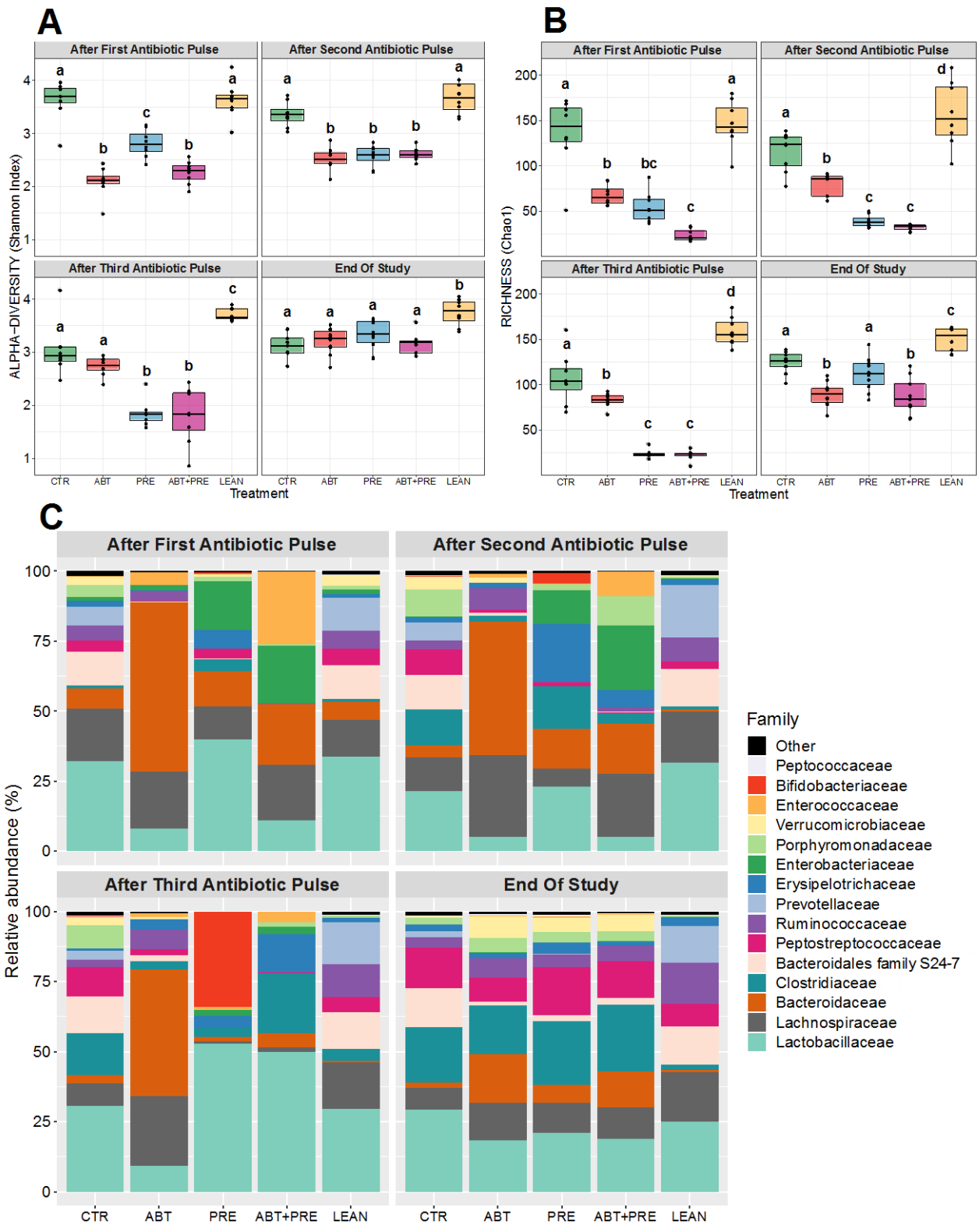

A
After First Antibiotic Pulse
After Second Antibiotic Pulse
After Third Antibiotic Pulse
End Of Study
ALPHA−DIVERSITY (Shannon Index)
CTR
ABT
PRE
ABT+PRE
LEAN
Treatment
B
RICHNESS (Chao1)
C
Relative abundance (%)
Family
Other
Peptococcaceae
Bifidobacteriaceae
Enterococcaceae
Verrucomicrobiaceae
Porphyromonadaceae
Enterobacteriaceae
Erysipelotrichaceae
Prevotellaceae
Ruminococcaceae
Peptostreptococcaceae
Bacteroidales family S24-7
Clostridiaceae
Bacteroidaceae
Lachnospiraceae
Lactobacillaceae

Figure 4.7 Between-group variations in alpha-diversity of gut bacterial communities and relative abundances of the 15 most abundant bacterial families in male rats over time.

(A and B) Shannon diversity (A) and Chao1 estimated richness (B) display between-group differences in alpha-diversity in male rats over time (n=8-10 rats per group/time point). ANOVA with Tukey post-hoc test; $P<0.05$. (C) Between-group relative abundances of the 15 most abundant bacterial families in male rats after the first, second, third antibiotic exposure and at the end of the study. Kruskal-Wallis test with Dunn post-hoc tests and Benjamin-Holmes False Discovery Rate (FDR) correction; $P<0.05$ (To see significant differences between groups: Table 4.2). CTR, control; ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; LEAN, lean control.

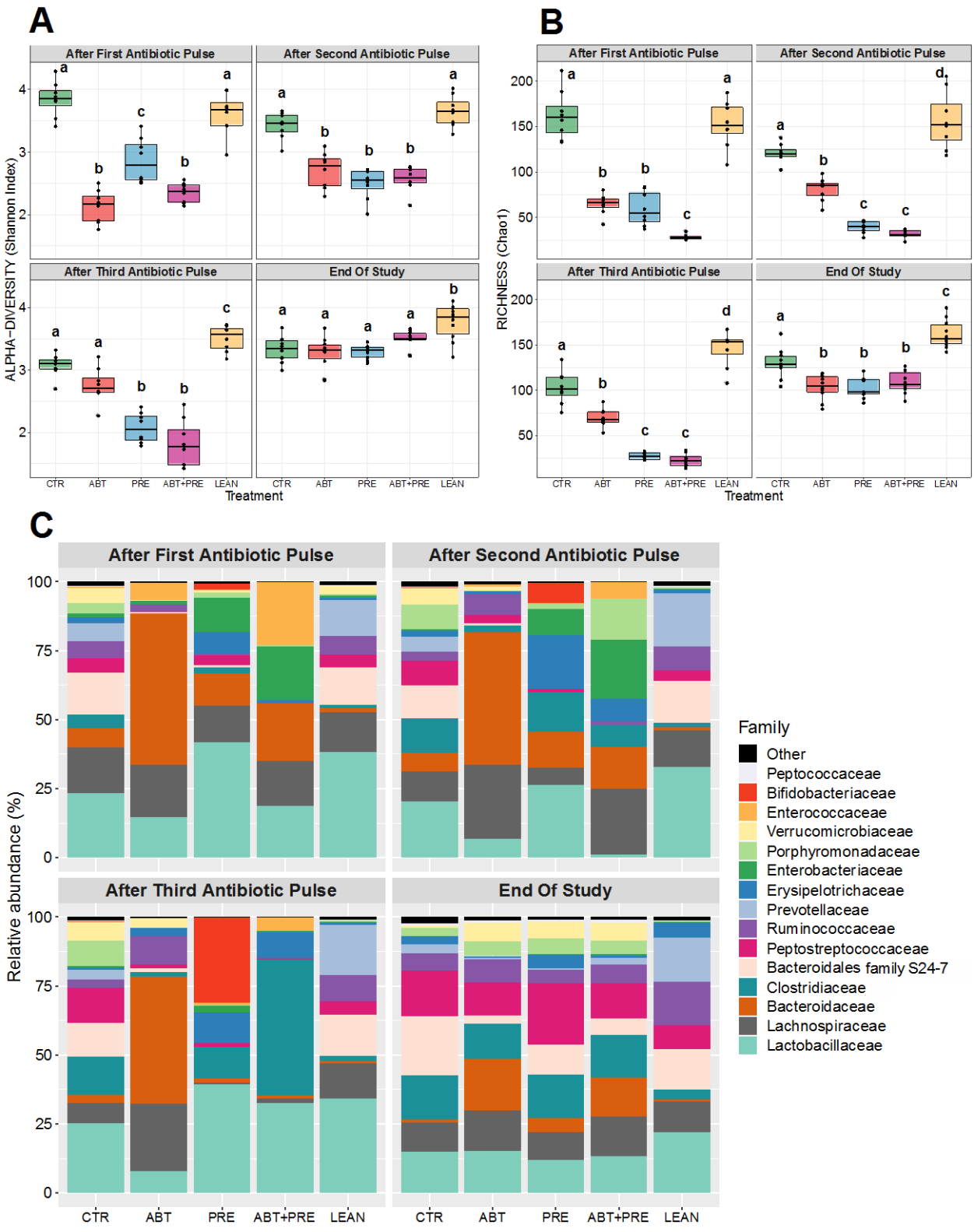

A
After First Antibiotic Pulse
After Second Antibiotic Pulse
After Third Antibiotic Pulse
End Of Study
ALPHA−DIVERSITY (Shannon Index)
CTR
ABT
PRE
ABT+PRE
LEAN
Treatment
B
RICHNESS (Chao1)
C
Relative abundance (%)
Family
Other
Peptococcaceae
Bifidobacteriaceae
Enterococcaceae
Verrucomicrobiaceae
Porphyromonadaceae
Enterobacteriaceae
Erysipelotrichaceae
Prevotellaceae
Ruminococcaceae
Peptostreptococcaceae
Bacteroidales family S24-7
Clostridiaceae
Bacteroidaceae
Lachnospiraceae
Lactobacillaceae

Figure 4.8 Between-group variations in alpha-diversity of gut bacterial communities and relative abundances of the 15 most abundant bacterial families in female rats over time.

(A and B) Shannon diversity (A) and Chao1 estimated richness (B) display between-group differences in alpha-diversity in female rats over time (n=8-10 rats per group/time point). ANOVA with Tukey post-hoc test; $P<0.05$. (C) Between-group relative abundances of the 15 most abundant bacterial families in female rats after the first, second, third antibiotic exposure and at the end of the study. Kruskal-Wallis test with Dunn post-hoc tests and Benjamin-Holmes False Discovery Rate (FDR) correction; $P<0.05$ (To see significant differences between groups: Table 4.3). CTR, control; ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; LEAN, lean control.

Table 4.2 Between-group changes in relative abundance of the 15 most abundant bacterial families in male rats after the first, second, third antibiotic exposure and at the end of the study.

- After first antibiotic exposure

Family/Group	CTR	ABT	PRE	ABT+PRE	LEAN
Peptococcaceae	0.002±0.000^{a}	0.000±0.000^{b}	0.000±0.000^{b}	0.000±0.000^{b}	0.002±0.000^{a}
Bifidobacteriaceae	0.000±0.000^{a}	0.000±0.000^{a}	0.004±0.002^{b}	0.000±0.000^{a}	0.000±0.000^{a}
Verrucomicrobiaceae	0.030±0.008^{a}	0.000±0.000^{b}	0.006±0.005^{b}	0.000±0.000^{b}	0.038±0.010^{a}
Enterococcaceae	0.001±0.000^{a}	0.046±0.010^{bc}	0.004±0.001^{ac}	0.261±0.022^{b}	0.000±0.000^{a}
Porphyromonadaceae	0.043±0.010^{a}	0.000±0.000^{b}	0.016±0.005^{ac}	0.006±0.004^{bc}	0.014±0.006^{ac}
Prevotellaceae	0.068±0.017^{a}	0.000±0.000^{b}	0.001±0.001^{b}	0.000±0.000^{b}	0.118±0.019^{a}
Erysipelotrichaceae	0.021±0.006^{a}	0.002±0.000^{b}	0.066±0.019^{a}	0.001±0.001^{b}	0.015±0.003^{a}
Enterobacteriaceae	0.014±0.003^{a}	0.018±0.008^{a}	0.175±0.031^{b}	0.205±0.025^{b}	0.016±0.004^{a}
Ruminococcaceae	0.051±0.007^{a}	0.035±0.005^{a}	0.001±0.001^{b}	0.002±0.001^{b}	0.063±0.011^{a}
Bacteroidales_S24-7_group	0.121±0.019^{a}	0.003±0.002^{b}	0.003±0.002^{b}	0.000±0.000^{b}	0.121±0.008^{a}
Peptostreptococcaceae	0.041±0.008^{a}	0.003±0.001^{b}	0.033±0.009^{a}	0.000±0.000^{b}	0.059±0.010^{a}
Clostridiaceae_1	0.011±0.003^{a}	0.000±0.000^{b}	0.044±0.011^{a}	0.000±0.000^{b}	0.010±0.002^{a}
Lachnospiraceae	0.187±0.036^{ab}	0.203±0.017^{a}	0.120±0.010^{b}	0.196±0.022^{ab}	0.130±0.019^{b}
Bacteroidaceae	0.071±0.016^{b}	0.605±0.027^{a}	0.126±0.026^{bc}	0.217±0.041^{ac}	0.066±0.029^{b}
Lactobacillaceae	0.323±0.042^{a}	0.081±0.012^{b}	0.400±0.025^{a}	0.111±0.025^{b}	0.337±0.049^{a}

- After second antibiotic exposure – Males

Family/Group	CTR	ABT	PRE	ABT+PRE	LEAN
Peptococcaceae	0.003±0.001^{a}	0.001±0.001^{b}	0.000±0.000^{b}	0.000±0.000^{b}	0.002±0.001^{a}
Bifidobacteriaceae	0.002±0.000^{acd}	0.000±0.000^{b}	0.039±0.019^{c}	0.000±0.000^{b}	0.001±0.000^{bd}
Verrucomicrobiaceae	0.045±0.014^{a}	0.018±0.014^{a}	0.000±0.000^{b}	0.000±0.000^{b}	0.003±0.001^{ab}
Enterococcaceae	0.000±0.000^{a}	0.018±0.006^{bc}	0.003±0.001^{ac}	0.088±0.029^{b}	0.001±0.001^{a}
Porphyromonadaceae	0.096±0.010^{a}	0.000±0.000^{b}	0.021±0.005^{c}	0.105±0.021^{a}	0.004±0.001^{bc}
Prevotellaceae	0.065±0.015^{a}	0.000±0.000^{b}	0.000±0.000^{b}	0.000±0.000^{b}	0.187±0.006^{a}
Erysipelotrichaceae	0.019±0.004^{a}	0.020±0.016^{a}	0.206±0.025^{b}	0.062±0.035^{a}	0.022±0.002^{a}
Enterobacteriaceae	0.003±0.002^{a}	0.000±0.000^{a}	0.121±0.016^{bc}	0.231±0.037^{b}	0.003±0.000^{ac}
Ruminococcaceae	0.033±0.006^{ac}	0.077±0.015^{a}	0.002±0.000^{b}	0.017±0.006^{bc}	0.086±0.015^{a}

Bacteroidales_S24-7_group	0.123±0.021[a]	0.012±0.004[b]	0.000±0.000[c]	0.000±0.000[c]	0.131±0.007[a]
Peptostreptococcaceae	0.089±0.008[a]	0.011±0.007[bc]	0.012±0.005[bc]	0.003±0.003[c]	0.029±0.008[ab]
Clostridiaceae_1	0.129±0.014[a]	0.021±0.012[b]	0.152±0.050[a]	0.037±0.016[b]	0.011±0.005[b]
Lachnospiraceae	0.120±0.024[ac]	0.291±0.026[b]	0.065±0.025[a]	0.224±0.024[b]	0.181±0.019[bc]
Bacteroidaceae	0.043±0.010[ac]	0.477±0.024[b]	0.142±0.022[a]	0.181±0.040[a]	0.010±0.003[c]
Lactobacillaceae	0.215±0.037[a]	0.051±0.019[b]	0.230±0.036[a]	0.051±0.050[b]	0.315±0.026[a]

- After third antibiotic exposure – Males

Family/Group	**CTR**	**ABT**	**PRE**	**ABT+PRE**	**LEAN**
Peptococcaceae	0.002±0.001[a]	0.000±0.000[b]	0.000±0.000[b]	0.000±0.000[b]	0.003±0.000[a]
Bifidobacteriaceae	0.002±0.001[a]	0.000±0.000[a]	0.338±0.015[b]	0.000±0.000[a]	0.001±0.000[a]
Verrucomicrobiaceae	0.030±0.012[a]	0.009±0.005[a]	0.000±0.000[b]	0.000±0.000[b]	0.000±0.000[b]
Enterococcaceae	0.000±0.000[a]	0.011±0.006[b]	0.010±0.003[b]	0.039±0.008[b]	0.000±0.000[a]
Porphyromonadaceae	0.083±0.014[a]	0.000±0.000[b]	0.001±0.001[bd]	0.015±0.012[bc]	0.006±0.001[acd]
Prevotellaceae	0.031±0.007[a]	0.000±0.000[b]	0.000±0.000[b]	0.000±0.000[b]	0.150±0.018[a]
Erysipelotrichaceae	0.007±0.002[a]	0.038±0.004[bc]	0.041±0.011[bc]	0.134±0.047[b]	0.016±0.002[ac]
Enterobacteriaceae	0.002±0.000[a]	0.000±0.000[a]	0.022±0.008[b]	0.026±0.011[b]	0.001±0.000[a]
Ruminococcaceae	0.029±0.009[ad]	0.069±0.011[ac]	0.000±0.000[b]	0.003±0.001[bd]	0.120±0.13[c]
Bacteroidales_S24-7_group	0.131±0.023[a]	0.021±0.005[a]	0.000±0.000[b]	0.000±0.000[b]	0.132±0.017[a]
Peptostreptococcaceae	0.104±0.012[a]	0.021±0.015[bc]	0.001±0.001[b]	0.003±0.002[b]	0.052±0.012[ac]
Clostridiaceae_1	0.149±0.024[a]	0.030±0.015[b]	0.031±0.017[b]	0.214±0.058[a]	0.041±0.014[b]
Lachnospiraceae	0.079±0.024[ac]	0.249±0.014[b]	0.008±0.005[a]	0.017±0.010[a]	0.165±0.011[bc]
Bacteroidaceae	0.030±0.007[a]	0.452±0.029[b]	0.017±0.007[a]	0.049±0.033[a]	0.006±0.002[a]
Lactobacillaceae	0.307±0.036[a]	0.092±0.036[b]	0.530±0.031[c]	0.499±0.082[ac]	0.300±0.037[a]

- End of Study – Males

Family/Group	**CTR**	**ABT**	**PRE**	**ABT+PRE**	**LEAN**
Peptococcaceae	0.004±0.000	0.006±0.001	0.004±0.001	0.004±0.000	0.003±0.000
Bifidobacteriaceae	0.000±0.000[a]	0.000±0.000[a]	0.000±0.000[a]	0.000±0.000[a]	0.000±0.000[a]
Verrucomicrobiaceae	0.005±0.004[a]	0.079±0.013[b]	0.050±0.009[b]	0.060±0.015[b]	0.000±0.000[a]
Enterococcaceae	0.000±0.000[a]	0.001±0.000[a]	0.005±0.003[a]	0.001±0.000[a]	0.000±0.000[a]
Porphyromonadaceae	0.023±0.003[ac]	0.051±0.008[b]	0.037±0.005[ab]	0.034±0.007[ab]	0.007±0.001[c]

Prevotellaceae	0.019±0.004[a]	0.000±0.000[b]	0.000±0.000[b]	0.001±0.000[b]	0.130±0.012[a]
Erysipelotrichaceae	0.015±0.006[a]	0.017±0.004[a]	0.040±0.010[a]	0.015±0.003[a]	0.032±0.013[a]
Enterobacteriaceae	0.001±0.000[ac]	0.003±0.000[b]	0.002±0.000[ab]	0.002±0.000[ab]	0.000±0.000[c]
Ruminococcaceae	0.040±0.005[a]	0.071±0.011[a]	0.046±0.007[a]	0.054±0.011[a]	0.147±0.018[b]
Bacteroidales_S24-7_group	0.138±0.013[a]	0.015±0.002[b]	0.021±0.010[b]	0.022±0.004[b]	0.136±0.021[a]
Peptostreptococcaceae	0.144±0.013[a]	0.084±0.012[bc]	0.172±0.012[a]	0.132±0.017[ac]	0.080±0.019[bc]
Clostridiaceae_1	0.198±0.016[a]	0.174±0.034[a]	0.226±0.020[a]	0.140±0.033[a]	0.019±0.007[b]
Lachnospiraceae	0.078±0.006[a]	0.135±0.017[ab]	0.109±0.014[ab]	0.113±0.018[ab]	0.178±0.026[b]
Bacteroidaceae	0.017±0.004[ad]	0.172±0.022[b]	0.064±0.011[cd]	0.128±0.011[bc]	0.008±0.002[a]
Lactobacillaceae	0.294±0.040	0.183±0.020	0.210±0.037	0.189±0.046	0.249±0.043

Values displayed are mean±SE (relative abundance). N=8-10 rats per group/time point. Kruskal-Wallis test with Dunn post-hoc tests and Benjamin-Holmes False Discovery Rate (FDR) correction; $P<0.05$. CTR, control; ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; LEAN, lean control.

Table 4.3 Between-group changes of relative abundances of 15 most abundant bacterial families in female rats after the first, second, third antibiotic exposure and at the end of the study.

- After first antibiotic exposure – Females

Family/Group	**CTR**	**ABT**	**PRE**	**ABT+PRE**	**LEAN**
Peptococcaceae	0.004±0.001[a]	0.000±0.000[b]	0.000±0.000[b]	0.000±0.000[b]	0.003±0.001[a]
Bifidobacteriaceae	0.000±0.000[a]	0.000±0.000[a]	0.022±0.011[b]	0.000±0.000[a]	0.000±0.000[a]
Enterococcaceae	0.004±0.002[a]	0.063±0.011[b]	0.002±0.001[a]	0.229±0.035[b]	0.001±0.000[a]
Verrucomicrobiaceae	0.056±0.017[a]	0.001±0.000[bd]	0.010±0.004[acd]	0.000±0.000[b]	0.030±0.011[c]
Porphyromonadaceae	0.037±0.008[a]	0.000±0.000[b]	0.018±0.007[ac]	0.006±0.003[bc]	0.005±0.002[bc]
Enterobacteriaceae	0.014±0.003[a]	0.014±0.005[a]	0.126±0.033[b]	0.193±0.030[b]	0.008±0.002[a]
Erysipelotrichaceae	0.021±0.004[a]	0.001±0.001[b]	0.078±0.013[a]	0.008±0.007[b]	0.009±0.001[b]
Prevotellaceae	0.065±0.013[a]	0.000±0.000[b]	0.001±0.001[b]	0.000±0.000[b]	0.131±0.010[a]
Ruminococcaceae	0.064±0.009[a]	0.024±0.004[ab]	0.003±0.003[b]	0.003±0.002[b]	0.068±0.011[a]
Peptostreptococcaceae	0.050±0.008[a]	0.002±0.000[b]	0.036±0.010[a]	0.001±0.001[b]	0.046±0.008[a]
Bacteroidales_S24-7_group	0.153±0.017[a]	0.007±0.003[b]	0.008±0.003[b]	0.000±0.000[b]	0.134±0.010[a]

Clostridiaceae_1	0.048±0.024[a]	0.000±0.000[b]	0.022±0.005[a]	0.000±0.000[b]	0.0122±0.003[a]
Bacteroidaceae	0.070±0.009[a]	0.548±0.031[b]	0.117±0.023[ac]	0.208±0.019[bc]	0.016±0.009[a]
Lachnospiraceae	0.168±0.019[a]	0.189±0.013[a]	0.132±0.016[a]	0.162±0.024[a]	0.143±0.013[a]
Lactobacillaceae	0.232±0.023[a]	0.147±0.036[a]	0.419±0.032[b]	0.188±0.050[a]	0.383±0.025[b]

- After second antibiotic exposure – Females

Family/Group	**CTR**	**ABT**	**PRE**	**ABT+PRE**	**LEAN**
Peptococcaceae	0.004±0.001[a]	0.000±0.000[b]	0.000±0.000[b]	0.000±0.000[b]	0.004±0.001[a]
Bifidobacteriaceae	0.001±0.000[a]	0.000±0.000[a]	0.071±0.028[b]	0.000±0.000[a]	0.000±0.000[a]
Enterococcaceae	0.001±0.001[ac]	0.009±0.004[b]	0.003±0.001[bc]	0.059±0.016[b]	0.000±0.000[a]
Verrucomicrobiaceae	0.060±0.017[a]	0.015±0.012[b]	0.000±0.000[b]	0.000±0.000[b]	0.001±0.001[b]
Porphyromonadaceae	0.089±0.010[a]	0.000±0.000[b]	0.019±0.004[c]	0.149±0.023[a]	0.005±0.001[bc]
Enterobacteriaceae	0.005±0.002[ac]	0.000±0.000[a]	0.094±0.014[bc]	0.215±0.036[b]	0.002±0.001[a]
Erysipelotrichaceae	0.022±0.004[a]	0.011±0.003[a]	0.195±0.035[b]	0.083±0.045[a]	0.015±0.035[a]
Prevotellaceae	0.056±0.015[a]	0.000±0.000[b]	0.000±0.000[b]	0.000±0.000[b]	0.191±0.009[a]
Ruminococcaceae	0.032±0.004[ac]	0.075±0.011[a]	0.002±0.001[b]	0.013±0.005[bc]	0.087±0.008[a]
Peptostreptococcaceae	0.089±0.006[a]	0.030±0.026[b]	0.009±0.003[bc]	0.000±0.000[c]	0.038±0.013[ab]
Bacteroidales_S24-7_group	0.120±0.014[ac]	0.009±0.003[ab]	0.000±0.000[b]	0.000±0.000[b]	0.153±0.010[c]
Clostridiaceae_1	0.125±0.021[a]	0.026±0.014[b]	0.144±0.022[a]	0.078±0.034[ab]	0.017±0.009[b]
Bacteroidaceae	0.068±0.018[ac]	0.477±0.029[b]	0.131±0.067[c]	0.152±0.043[c]	0.009±0.002[a]
Lachnospiraceae	0.109±0.017[a]	0.270±0.017[b]	0.064±0.016[a]	0.238±0.031[b]	0.135±0.016[a]
Lactobacillaceae	0.203±0.002[ac]	0.067±0.016[bc]	0.262±0.056[a]	0.012±0.004[b]	0.3270.018[a]

- After third antibiotic exposure – Females

Family/Group	**CTR**	**ABT**	**PRE**	**ABT+PRE**	**LEAN**
Peptococcaceae	0.003±0.001[a]	0.000±0.000[b]	0.000±0.000[b]	0.000±0.000[b]	0.002±0.000[a]
Bifidobacteriaceae	0.001±0.001[a]	0.000±0.000[a]	0.308±0.047[b]	0.000±0.000[a]	0.000±0.000[a]
Enterococcaceae	0.000±0.000[a]	0.001±0.000[ab]	0.011±0.003[bc]	0.050±0.0131[c]	0.000±0.000[a]
Verrucomicrobiaceae	0.068±0.027[a]	0.035±0.015[a]	0.000±0.000[b]	0.000±0.000[b]	0.001±0.001[b]
Porphyromonadaceae	0.093±0.022[a]	0.000±0.000[b]	0.001±0.001[b]	0.001±0.000[b]	0.007±0.001[a]
Enterobacteriaceae	0.006±0.002[bc]	0.000±0.000[a]	0.024±0.006[b]	0.004±0.001[ab]	0.001±0.000[ac]
Erysipelotrichaceae	0.008±0.001[a]	0.029±0.006[a]	0.112±0.026[a]	0.092±0.016[a]	0.008±0.002[a]

Prevotellaceae	0.036±0.003[a]	0.000±0.000[b]	0.000±0.000[b]	0.000±0.000[b]	0.182±0.010[a]
Ruminococcaceae	0.028±0.006[ab]	0.1503±0.015[a]	0.001±0.000[b]	0.004±0.001[b]	0.094±0.016[a]
Peptostreptococcaceae	0.127±0.015[a]	0.011±0.008[b]	0.014±0.006[bc]	0.002±0.001[b]	0.050±0.017[ac]
Bacteroidales_S24-7_group	0.123±0.019[ab]	0.014±0.002[b]	0.000±0.000[c]	0.000±0.000[c]	0.150±0.007[a]
Clostridiaceae_1	0.138±0.019[ac]	0.018±0.013[b]	0.112±0.038[ad]	0.493±0.056[c]	0.019±0.012[bd]
Bacteroidaceae	0.030±0.009[a]	0.459±0.017[b]	0.016±0.010[a]	0.013±0.005[a]	0.008±0.002[a]
Lachnospiraceae	0.073±0.008[ac]	0.245±0.019[b]	0.006±0.002[a]	0.016±0.008[a]	0.128±0.015[bc]
Lactobacillaceae (Adjusted p value between C and A =0.06)	0.253±0.030[ac]	0.079±0.020[bc]	0.394±0.047[a]	0.326±0.045[a]	0.342±0.038[a]

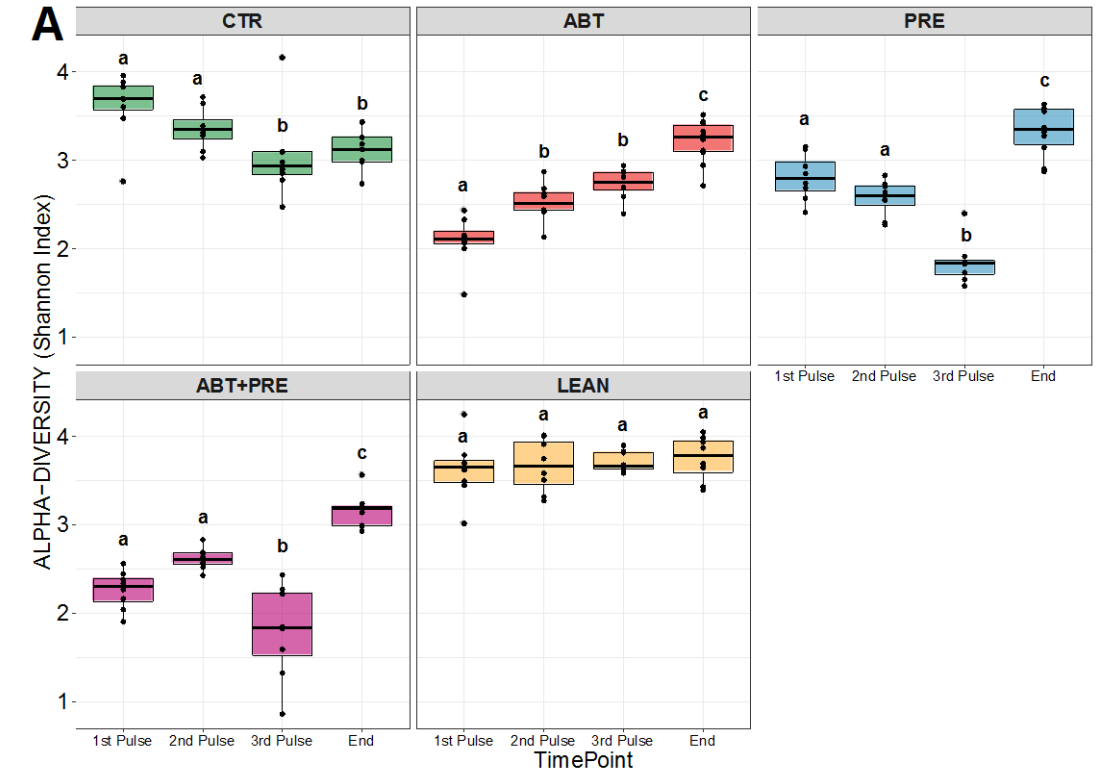
- End of Study – Females

Family/Group	**CTR**	**ABT**	**PRE**	**ABT+PRE**	**LEAN**
Peptococcaceae	0.007±0.001[ab]	0.008±0.001[a]	0.007±0.001[ab]	0.009±0.001[a]	0.004±0.000[b]
Bifidobacteriaceae	0.001±0.000[a]	0.000±0.000[a]	0.000±0.000[a]	0.000±0.000[a]	0.000±0.000[a]
Enterococcaceae	0.002±0.001[a]	0.000±0.000[b]	0.000±0.000[b]	0.002±0.001[a]	0.000±0.000[b]
Verrucomicrobiaceae	0.007±0.003[a]	0.068±0.015[b]	0.062±0.018[b]	0.066±0.014[b]	0.000±0.000[a]
Porphyromonadaceae	0.030±0.005[a]	0.051±0.011[a]	0.057±0.005[a]	0.048±0.009[a]	0.003±0.001[b]
Enterobacteriaceae	0.001±0.000[a]	0.001±0.000[a]	0.002±0.001[a]	0.003±0.001[a]	0.001±0.000[a]
Erysipelotrichaceae	0.029±0.006[a]	0.007±0.002[b]	0.048±0.008[a]	0.009±0.002[b]	0.054±0.025[a]
Prevotellaceae	0.032±0.008[ac]	0.004±0.004[b]	0.007±0.004[b]	0.024±0.016[ab]	0.160±0.020[c]
Ruminococcaceae	0.064±0.008[a]	0.086±0.013[a]	0.048±0.007[a]	0.069±0.006[a]	0.159±0.018[b]
Peptostreptococcaceae	0.164±0.015[ab]	0.117±0.017[b]	0.223±0.006[a]	0.126±0.020[b]	0.087±0.024[b]
Bacteroidales_S24-7_group	0.214±0.014[a]	0.032±0.005[b]	0.107±0.026[bc]	0.060±0.012[b]	0.146±0.012[ac]
Clostridiaceae_1	0.160±0.016[a]	0.127±0.025[a]	0.158±0.017[a]	0.155±0.023[a]	0.034±0.015[b]
Bacteroidaceae	0.012±0.002[ac]	0.187±0.014[b]	0.052±0.006[cd]	0.141±0.021[bd]	0.010±0.003[a]
Lachnospiraceae	0.105±0.016[a]	0.147±0.016[a]	0.102±0.012[a]	0.144±0.009[a]	0.111±0.017[a]
Lactobacillaceae	0.150±0.024[ab]	0.151±0.035[ab]	0.119±0.015[a]	0.134±0.014[ab]	0.222±0.022[b]

Values displayed are mean±SE (relative abundance). N=8-10 rats per group/time point. Kruskal-Wallis test with Dunn post-hoc tests and Benjamin-Holmes False Discovery Rate (FDR) correction; $P<0.05$. CTR, control; ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; LEAN, lean control.

4.3.4 Treatment disrupts gut bacterial community maturation in males and females

Considering microbial communities that reside in the gut, environmental perturbations (i.e. antibiotics) and lack of microbiota stability in the first three years of life[233], we decided to investigate microbial maturation/development for each experimental group separately over time (Figure 4.9 – 4.12). Antibiotic male (Figure 4.10) and female (Figure 4.12) gut microbiota development was disrupted with antibiotics, demonstrated by the bloom in *Bacteroidaceae* after each antibiotic pulse that contrasted with the control groups where levels remained relatively stable over time. Furthermore, *Lactobacillaceae* levels decreased over time in control/lean control groups in males (Figure 4.10) and females (Figure 4.12), peaking immediately after weaning (1st pulse). However, in antibiotic groups, the trend was the opposite as the highest levels of *Lactobacillaceae* were seen at the end of study, especially in males (Figure 4.10). *Bacteroidales* family *S24-7* and *Peptostreptococcaceae* were completely depleted in antibiotic/prebiotic groups during the intervention (1st – 3rd pulse) in males (Figure 4.10) and females (Figure 4.12) and levels increased only at the end of the study. On the contrary, control/lean control groups had higher levels of *Bacteroidales* family *S24-7* and *Peptostreptococcaceae* and these taxa were stable over time (1st pulse – end of the study). Prebiotic group's (PRE) gut microbiota development differed from control and antibiotic groups (CTR/LEAN/ABT) as higher *Bifidobacteriaceae* levels were seen after the 3rd pulse in males (Figure 4.10) and females (Figure 4.12). However, when prebiotics were co-administered with antibiotics (ABT+PRE), a bloom in *Clostridiaceae* was seen instead in males (Figure 4.10) and females (Figure 4.12). Interestingly, while a HFS diet in control (CTR) and prebiotic groups (PRE and ABT+PRE) decreased α-diversity over time (from 1st until 3rd pulse) in males (Figure 4.9A and 4.9B) and females (Figure 4.11A and 4.11B), the opposite occurred in ABT groups as α-diversity increased.


A
CTR
ABT
PRE
ABT+PRE
LEAN
ALPHA−DIVERSITY (Shannon Index)
1st Pulse
2nd Pulse
3rd Pulse
End
TimePoint

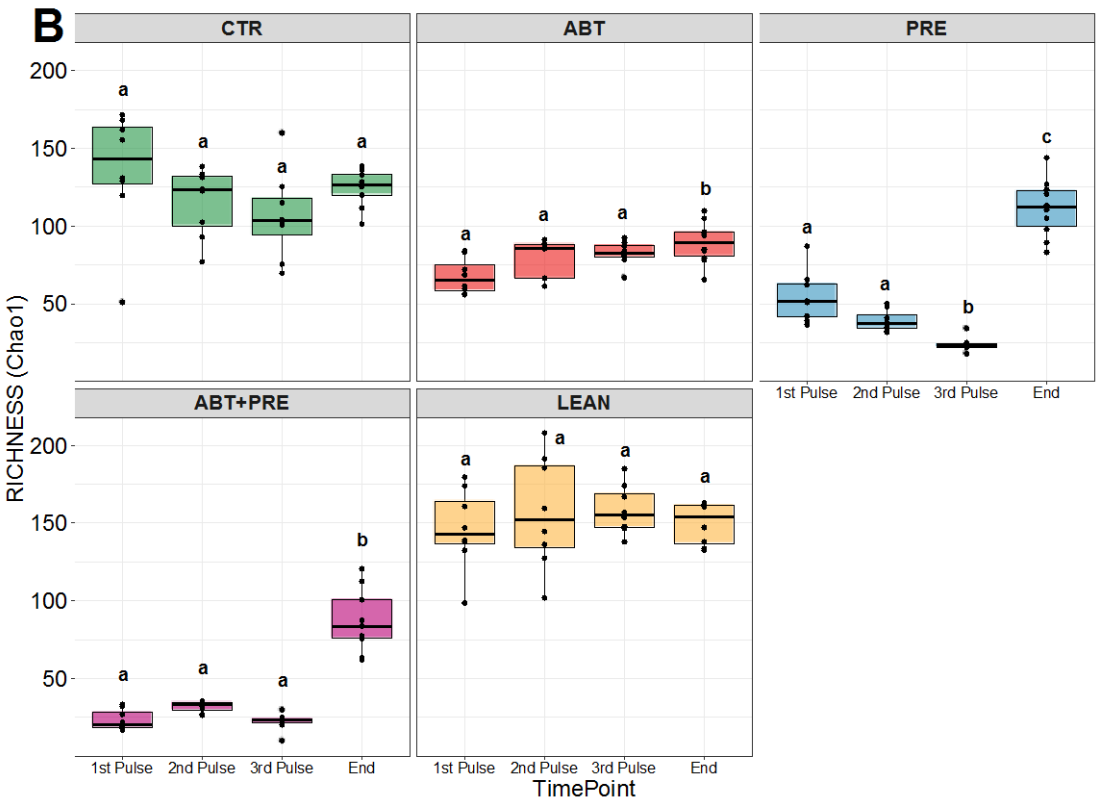

B
CTR
ABT
PRE
ABT+PRE
LEAN
RICHNESS (Chao1)
1st Pulse
2nd Pulse
3rd Pulse
End
TimePoint

Figure 4.9 Within-group differences in alpha-diversity over time in males

(A and B) Shannon diversity (A) and Chao1 estimated richness (B) (n=8-10 rats per group/time point). ANOVA with Tukey post-hoc test. CTR, control; ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; LEAN, lean control. 1st Pulse, After first antibiotic pulse; 2nd Pulse, After second antibiotic pulse; 3rd Pulse, After third antibiotic pulse; End, End of Study.

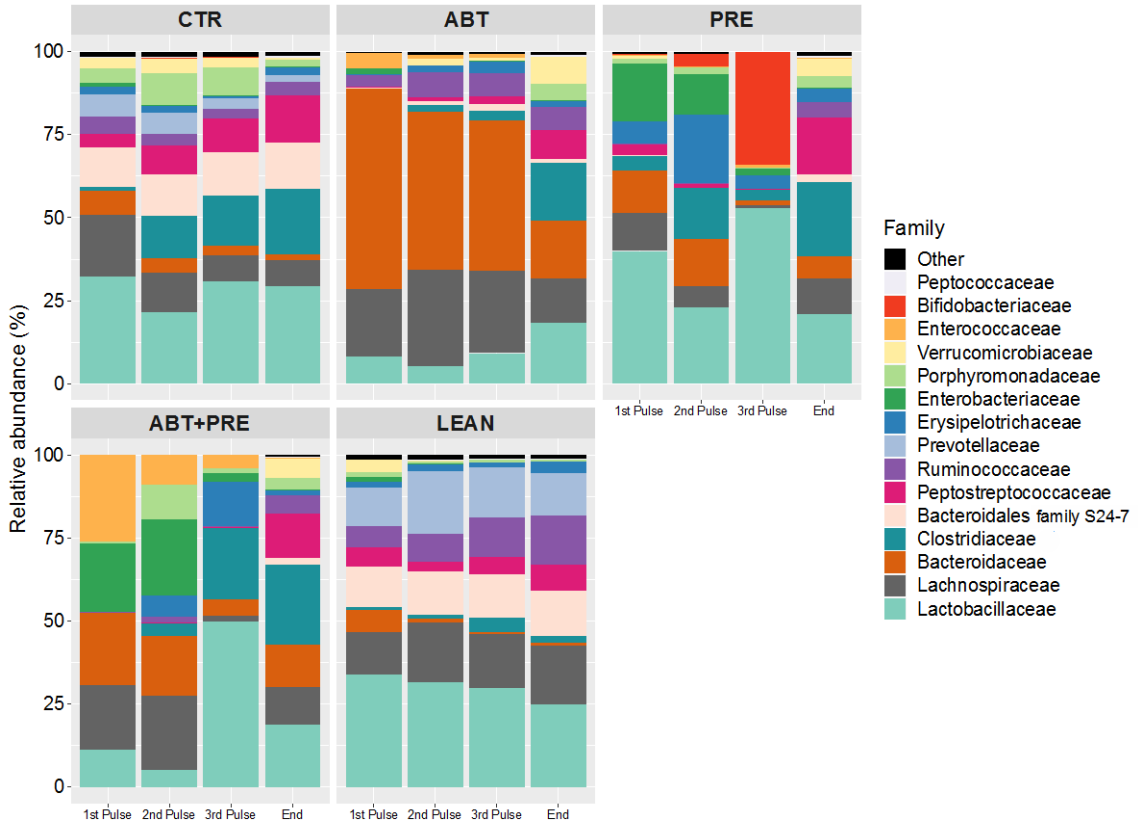
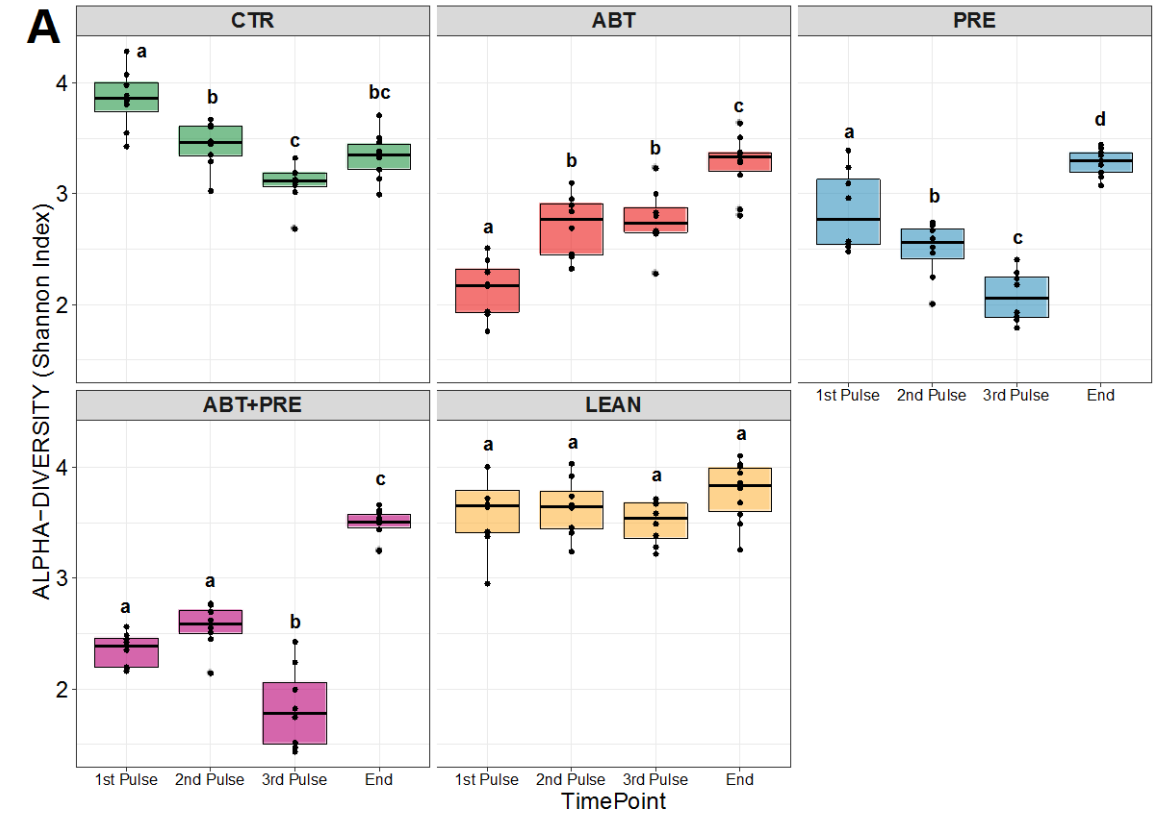


Figure 4.10 Within-group differences in relative abundance of the 15 most abundant bacterial families in males over time

n=8-10 rats per group/time point. CTR, control; ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; LEAN, lean control. 1st Pulse, After first antibiotic pulse; 2nd Pulse, After second antibiotic pulse; 3rd Pulse, After third antibiotic pulse; End, End of Study.


A
CTR
ABT
PRE
ABT+PRE
LEAN
ALPHA−DIVERSITY (Shannon Index)
1st Pulse
2nd Pulse
3rd Pulse
End
TimePoint

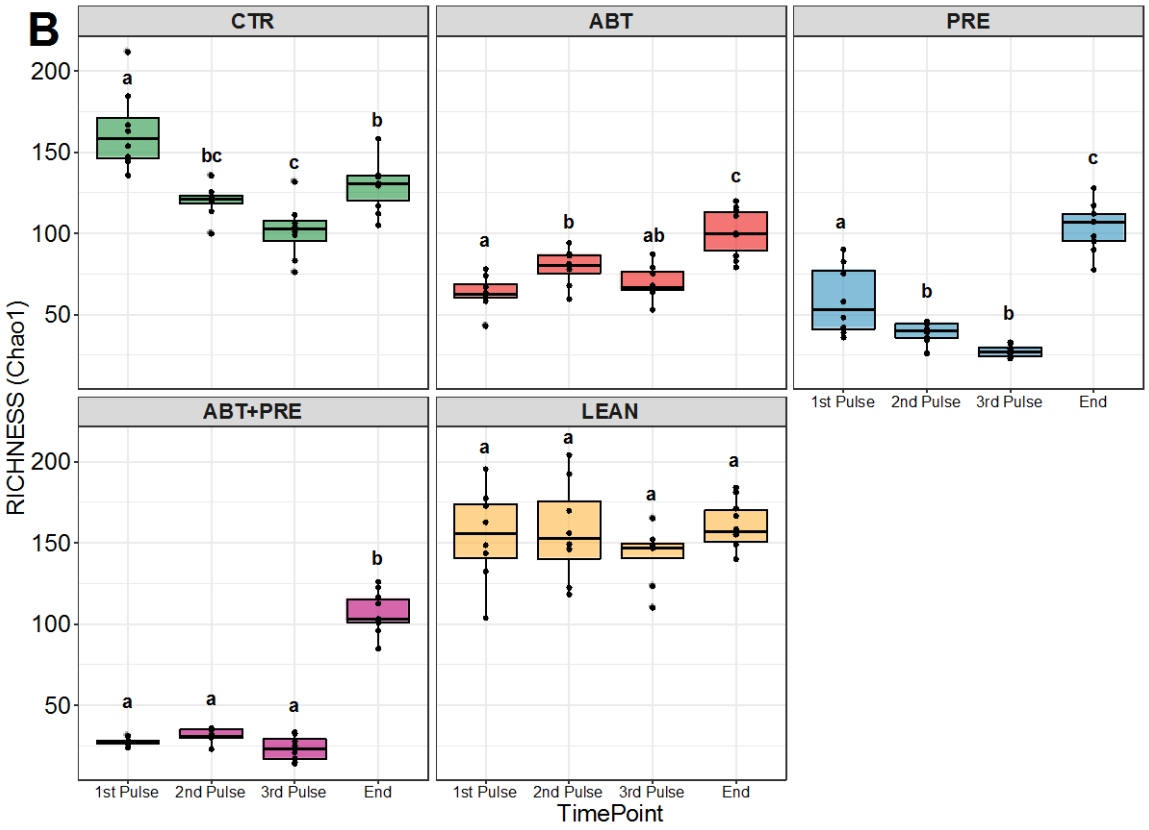

B
CTR
ABT
PRE
ABT+PRE
LEAN
RICHNESS (Chao1)
1st Pulse
2nd Pulse
3rd Pulse
End
TimePoint

Figure 4.11 Within-group differences in alpha-diversity over time in females

(A and B) Shannon diversity (A) and Chao1 estimated richness (B) (n=8-10 rats per group/time point). ANOVA with Tukey post-hoc test. CTR, control; ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; LEAN, lean control. 1st Pulse, After first antibiotic pulse; 2nd Pulse, After second antibiotic pulse; 3rd Pulse, After third antibiotic pulse; End, End of Study.

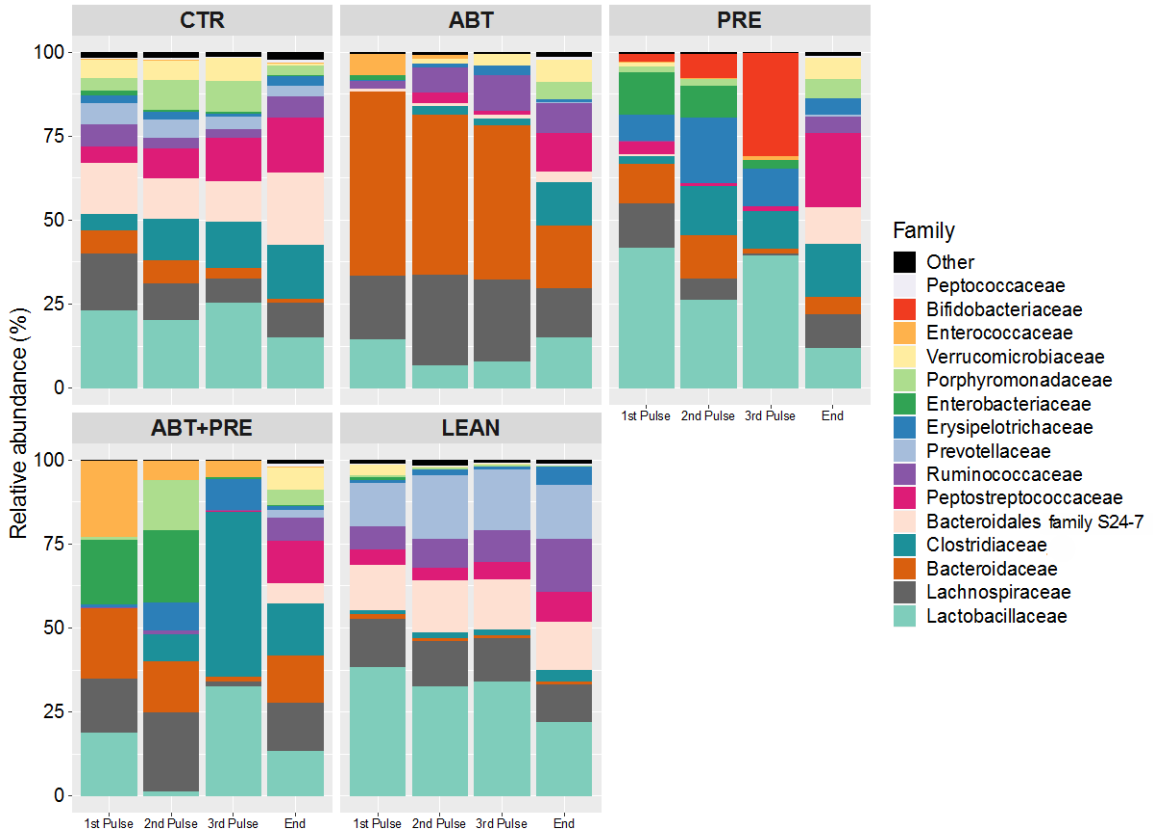


Figure 4.12 Within-group differences in relative abundance of the 15 most abundant bacterial families in females over time

n=8-10 rats per group/time point. CTR, control; ABT, antibiotic; PRE, prebiotic; ABT+PRE, antibiotic+prebiotic; LEAN, lean control. 1st Pulse, After first antibiotic pulse; 2nd Pulse, After second antibiotic pulse; 3rd Pulse, After third antibiotic pulse; End, End of Study.

4.5 Discussion

Antibiotics are life-saving drugs, but only recently has the impact of early-life antibiotic treatment on gut microbiota development and its metabolic consequences been described[130,147]. Here we investigated a therapeutic dose of an antibiotic class most commonly used in human children with the administration regimen mimicking that used in pediatric populations[234]. In our study, pulsed-antibiotic azithromycin treatment early in life affected host body weight, body composition, insulin resistance, hepatic and hypothalamic gene expression, as well as gut microbiota even after the antibiotic treatments were stopped. Although the negative effects of pulsed therapeutic-dose antibiotic treatment have been described[147], our study provides novel insight into the role of prebiotics in mitigating the adverse effects of early postnatal antibiotic treatment on weight gain and insulin resistance.

Our study demonstrates that early life pulsed antibiotic exposure (PAT) changes the capacity of the animals to respond to stressors such as a HFS diet, a finding reported previously[147]. Notably, antibiotic increased body weight in males and females at the end of the study, with males having significantly increased fat mass compared to all other groups. While Nobel *et al.* (2015)[147] investigated PAT in young female mice, our study included both male and female rats and demonstrated a stronger phenotype in males after PAT than females. Our results are in line with previous studies employing sub-therapeutic antibiotic exposures in animals as well as human studies, where boys/males[18,20,130,141,154,155,165] were more prone to obesity upon early-life antibiotic exposure. These studies showed that adverse effects seen in males are rarely mirrored in females and the reasons are poorly understood. Given that females respond differently to environmental stressors such as diet/physical activity/stress[235,236], it is likely that the response to antibiotics is also sex-specific. Several mechanisms have been proposed by others to explain the weight gain observed after early life antibiotic exposure[3]. Specifically, bacteria increase their energy harvesting capacity from diet, the number of health promoting bacteria decreases, metabolic signalling changes, hepatic lipogenesis increases, intestinal permeability increases and immune defense is impaired after antibiotic exposure[3].

In addition to the host physiology, PAT also had a major impact on the gut microbial ecosystem. As shown previously in female mice[147], a reduction in alpha-diversity (richness and Shannon index) was seen immediately after the first PAT, but the differences between groups were minimized by the end of study in both sexes due to the strong impact of HFS diet on microbial composition. In addition, our beta-diversity analysis (PCoA plots) showed progressive separation of antibiotic animals from controls with each PAT, indicating that the alterations to microbial composition were driven by antibiotics and are cumulative through time. We hypothesise that low alpha-diversity in combination with altered beta-diversity is due to the extremely high abundance of *Bacteroidaceae* after PAT, separating PAT animals from other groups. Furthermore, PAT changed the typical microbiota responses to a HFS diet, a finding also reported previously[147]. The typical response to a HFS diet would be an increase in Firmicutes at the expense of Bacteroidetes with a simultaneous decrease in microbial diversity[50]. But in our PAT animals, a spike in *Bacteroidaceae* (Bacteroidetes) and depletion in *Lactobacillaceae* (Firmicutes) with a concurrent increase in alpha-diversity was seen during the PAT pulses and the HFS diet consumption. One possible explanation is the mechanism of action of azithromycin; since it largely targets Gram-positive bacteria[237] such as Firmicutes, an opportunistic bloom of Gram-negatives (Bacteroidetes) could be expected. Depleting *Lactobacillus* in PAT animals early in life might partially explain increased weight gain at the end of study in both sexes as low levels of *Lactobacillus* present during the developmental window in another study led to increased adiposity/weight later in life[130].

A critical element of a healthy microbiota is ecosystem stability which is defined by the ability of the community to remain unchanged during perturbations (resistance) and the capacity to return to initial state after the insult (resilience).[10] At birth, a simple gut bacterial community is established with low diversity, low bacterial load and low resilience[10]. While minor fluctuations to a certain extent are expected, major pulsed perturbations such as antibiotics or continuous perturbations (HFS diet) may result in an ill-defined state of the intestinal microbial community (dysbiosis) contributing to disease, especially early in life when the gut microbiota is still being established.[10] The so-called "insurance hypothesis" defines a strong relationship between microbial diversity and

ecosystem stability meaning that communities containing many species have greater capacity to return to a stable equilibrium after the insult[10]. Furthermore, development of gut microbiota is directional indicating that the growth of certain species is dependent on the presence of other species (structured temporal succession). For example, the presence of oxygen in the intestine after birth promotes the growth of facultative anaerobic bacteria (i.e. *Lactobacillus* spp.) until the oxygen reserves are depleted and then anaerobic bacteria (i.e. Bacteroidetes) replace them[10]. In our study, the opposite was observed in antibiotic animals with high levels of anaerobic *Bacteroidaceae* and low levels of *Lactobacillaceae* demonstrating altered establishment of gut microbiota. Furthermore, *Bacteroidales* family *S24-7* and *Peptostreptococcaceae* were under-represented in ABT groups when compared to controls over time. Interestingly, similar microbial profiles as in our antibiotic-treated animals were seen in a colitis mouse model. Expansion of *Bacteroidaceae* and depletion of commensal bacteria *Bacteroidales* family *S24-7* and *Lactobacillus* species was reported in mice with colitis[238], further suggesting dysbiotic maturation of gut microbiota in our ABT animals. In line with this, it was previously reported that individuals with low bacterial richness and *Bacteroides*-dominated community had increased inflammatory markers, lower functional redundancy and lower resistance[239]. In our study, ABT males and females had *Bacteroides*-dominated intestinal communities in combination with low richness. It is therefore possible that microbial development disruptions with antibiotics early in life contributed to metabolic impairments (insulin resistance) observed in antibiotic animals. In humans, antibiotic use in the first year of life was associated with metabolic syndrome later in life[240] and unfortunately, the highest antibiotic use seen in humans occurs in the first two years of life.[241]

Beyond the phenotypic and gut microbiota changes, we also observed metabolic impairments after PAT, mostly in males. Increased fasting insulin levels/insulin resistance were seen at the end of the pulses and the end of the study in PAT males. It is likely that the insulin resistance was worse in males because of the low expression of insulin receptor substrates. Specifically, we saw lower hepatic insulin receptor-2 (IRS-2) expression at the end of the antibiotic pulses in PAT males when compared to controls, but we did not observe this trend in females. Similarly, PAT males had a trend towards

lower hypothalamic IRS-1/2 expression, whereas females did not. As reviewed previously[3], another putative mechanism for increased insulin resistance seen after antibiotic exposure could be metabolic endotoxemia. The authors suggest that increased inflammation caused by translocation of lipopolysaccharide (LPS) from Gram-negative bacteria present in the gut leads to systemic inflammation and consequently to insulin resistance[3]. It is known that pro-inflammatory cytokines such as interleukin-6 and/or tumor necrosis factor–α (TNF-α) interfere with insulin signalling[242], thereby reducing insulin sensitivity. Furthermore, high-fat/high-carbohydrate diets increase gut inflammation and permeability[243], thus enabling increased LPS translocation to the blood stream. Increased plasma LPS along with greater gut permeability leads to inflammation, weight gain, fasting hyperglycaemia and hyperinsulinemia[244]. Since male PAT animals in our study had ≈10x greater fecal relative abundance of Gram-negative *Bacteroidaceae* (a source of LPS) and were on a HFS diet (increased gut permeability), it is possible that higher levels of LPS in our ABT group contributed to low-grade inflammation, increased fat mass and insulin resistance. Interestingly, LPS levels at the end of the study in males (Figure 4.2B) mirrored body fat levels detected in those animals (Figure 4.1E). Prebiotic administration in our study reduced body weight and fat mass, improved insulin sensitivity, modified gut microbiota composition and altered hepatic/hypothalamic gene expression. A possible mechanism for these benefits is the ability of prebiotics to increase the production of GLP-2 which in turn improves intestinal barrier integrity, insulin sensitivity, inflammatory markers and reduces oxidative stress[245]. Chronic administration of GLP-2 to *ob/ob* mice decreased plasma LPS level by 50%, lowered plasma inflammation markers and increased the expression of tight junction proteins (ZO-1 and occludin)[245]. In another study[206] OFS supplementation improved intestinal permeability, decreased LPS levels and consequently led to lower body weight and fat mass. We confirm lower LPS levels and fat mass in males in our prebiotic group with a trend towards a decrease in ABT+PRE group. Interestingly, these health-promoting effects of OFS supplementation were abrogated once *Lactobacillus* and *Bifidobacterium* growth was inhibited with antibiotics[206]. It is possible that the positive effects of OFS supplementation in our study was a consequence of increased levels of *Lactobacillaceae*, which were depleted after PAT. In line with this, other studies showed that probiotic

strains such as *Lactobacillus rhamnosus* GG and *Lactobacillus casei* DN-114-001 improve epithelial barrier function via tight junction proteins[246,247]. In addition, prebiotics also improve gut permeability due to changes in the morphology of the intestine by increasing the villus height, crypt depth and thickening the mucus layer[245]. During the fermentation of prebiotics, butyrate is produced, feeding colonocytes and increasing the mucus layer[245]. Increased cecum size is a marker of increased bacterial fermentation[206,248] which was clearly evident in our prebiotic animals in both males and females. Taken together, we believe improvements in insulin sensitivity in our prebiotic groups might be partially explained by increased levels of *Lactobacillaceae*, normalization of extremely high levels of *Bacteroidaceae* (source of LPS) and improved gut permeability seen by lower LPS levels in prebiotic groups.

Besides gut microbiota/body composition/metabolic changes, prebiotics also changed hepatic and hypothalamic gene expression. Specifically, reductions in hypothalamic IRS-1/2 gene expression seen in PAT males were normalized with prebiotics and could further explain improvements in insulin sensitivity seen in prebiotic males. Of note, it was shown previously[248] that prebiotic can modify hypothalamic expression of genes relevant to anxiety and the authors propose that changes were mediated by SCFAs. To our knowledge, we are the first to show that early life prebiotic supplementation added to PAT improves hypothalamic expression but is not able to correct hepatic IRS expression in males. Nevertheless, since hypothalamic IR expression plays a major role in energy homeostasis (IR deficiency led to increased body weight and adiposity[249]), we hypothesize that increasing hypothalamic IRS with prebiotics promoted leanness in our study, at least in part.

While prebiotics reduced insulin/insulin resistance, leptin and glucose in both sexes, normalization to the levels of lean controls was only seen in females. We speculate that extremely low leptin, insulin and glucose in prebiotic females in turn overexpressed NPY/AgRP neurons as NPY/AgRP expression is driven by these peripheral hormonal signals[250]. In a healthy state, fasting in combination with low insulin, leptin and glucose leads to increased hypothalamic NPY/AgRP expression and has no impact on POMC peptides[250], exactly what was observed in our prebiotic females. Since these hypothalamic circuits are the most important regulators of energy homeostasis and food

intake, it is likely that only females trended toward lower food intake during PAT since their feeding neuronal pathways were intact and not blunted by a HFS.

Taken together, we show that direct pulsed administration of azithromycin to young rats impairs microbiota composition/maturation, their body weight, fat mass, LPS levels, hepatic/hypothalamic gene expression and insulin production/sensitivity in a sex-specific manner. In a novel and mechanistic experiment, we demonstrate that the unfavorable outcomes of antibiotics are prevented with prebiotic co-administration even when the doses administered are therapeutic and mimic administration of antibiotics in pediatric populations. It is unknown whether this non-invasive intervention with prebiotics translates to human children, but the potential to reduce harm of early life antibiotic exposure is promising.

4.6 Acknowledgments

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4.7 Author Contributions

Teja Klancic and Raylene A. Reimer conceived the experiments. Teja Klancic, Jolene Wong, Ashley Choo, Jodi E. Nettleton and Faye Chleilat carried out the experiments. Isabelle Laforest-Lapointe, Marie-Claire Arrieta and Teja Klancic performed gut microbiota bioinformatics. Teja Klancic performed all other data analysis. Teja Klancic drafted the paper and all authors critically revised the manuscript for important intellectual content. Raylene A. Reimer received funding and had primary responsibility as the study supervisor.

CHAPTER FIVE: ANTIBIOTICS GIVEN DURING LABOR AND BIRTH INCREASES BODY MASS INDEX *z* SCORES IN CHILDREN AT ONE YEAR OF AGE: RESULTS FROM THE ALL OUR FAMILIES (AOF) PREGNANCY COHORT

5.1 Abstract

Background: Increased risk of obesity with early life antibiotic exposure has been reported in animal and human studies, however knowledge about intrapartum (during birth) antibiotic prophylaxis (IAP) and early life obesity risk is limited. Our objective was to determine whether maternal antibiotic exposure during birth (IAP, objective 1) and/or during pregnancy (objective 2) is associated with child body mass index (BMI) *z* scores in the first three years of life (calculated according to 2006 WHO standards).

Methods: Between May 2008 and May 2010, 3387 pregnant women were recruited for the prospective All Our Families (AOF) pregnancy cohort. For the current analysis we only included women with available data on antibiotic usage during birth (intrapartum (IAP); n=1303) and during pregnancy (n=1943). Maternal exposure to antibiotics during birth was obtained from obstetrical/birth records and self-reported by mothers via questionnaires during pregnancy. The primary outcomes of this study were infant BMI *z* scores at 1, 2 and 3 years of age, assessed according to WHO reference standards.

Results: A total of 441 women out of 1303 had IAP exposure while 187 out of 1943 women reported antibiotic use during pregnancy. One year old children born to mothers that had IAP had higher mean [SE] BMI *z* score (1.317 [0.194] unit) compared to infants with no maternal IAP exposure (0.755 [0.130] unit). After controlling for additional covariates such as gestational weight gain and pregnancy term status, intrapartum antibiotic exposure was associated with a 0.567 unit increase in BMI *z* score at 1 year of age (adjusted 95% CI, 0.094-1.039). Results were confirmed following sensitivity analysis. On the other hand, no association was found between antibiotic exposure during pregnancy and infant BMI *z* score at one, two or three years of age.

Conclusions: To our knowledge, this is the first time that an association has been shown between maternal intrapartum antibiotics and increased infant BMI *z* score at 1 year of age. Confirmation of this finding in other large pregnancy cohorts and investigation of interventions to mitigate the associated risk of obesity is warranted.

5.2 Background

Since the discovery of penicillin almost 100 years ago, antibiotics have revolutionized medicine by markedly reducing morbidity and mortality [251]. They are the most commonly prescribed medications to children [252] and while prescription rates vary greatly around the world, North America has one of the highest antibiotic uses in part because of their routine use during the birthing process [253]. More than 50% of pregnant women in the USA receive antibiotics during pregnancy [39] and >40% of newborns are given antibiotics either directly or indirectly through intrapartum antibiotic prophylaxis (IAP) [253]. The main indications for IAP are prevention of maternal infection during cesarean-section birth [14] and inhibition of the vertical transmission of group B streptococcus (GBS) during labor and delivery. GBS, which is carried by 10-30% of pregnant women, can cause an invasive infection that has ~50% infant mortality rate [254]. Guidelines for IAP administration have been devised to reduce vaginal bacterial count at delivery, protect the amniotic fluid compartment from GBS and achieve sufficiently high levels of the antibiotic in the fetal bloodstream [254]. In addition to IAP, on average, a child in the USA receives nearly three courses of antibiotics by the age of two years, about 10 courses by the age of 10 years and approximately 17 courses by 20 years of age [21].

A growing body of evidence from animal and epidemiological studies suggests that early-life antibiotic exposure increases the risk of several common diseases including allergies, asthma, inflammatory bowel disease, arthritis and obesity [255]. The linkage between early life antibiotic exposure and obesity risk has been demonstrated convincingly in causal studies in animals with weaker association data available from human studies [3,252]. A large meta-analysis involving 445,880 participants suggested an increased risk of childhood overweight (relative risk [RR] = 1.23, 95% confidence interval [CI] = 1.13-1.35, $P<0.001$) and obesity (RR=1.21, 95% CI = 1.13-1.30, $P<0.001$) when antibiotics were administered early in life (before birth/first two years of life) [256]. In addition, each additional antibiotic course increased the risk of overweight/obesity by 6%/7%, respectively, showing a dose-response relationship between antibiotic exposure and adiposity [256]. A multicenter cohort study with 43,332 children reported a strong link between prenatal antibiotic exposure and increased risk of childhood obesity at age seven years with second trimester antibiotic exposure having the strongest impact [257]. Similarly,

a study of 436 mother-child pairs reported an 84% increased risk of obesity in children at 7 years of age when their mothers received antibiotics in the second or third trimester of pregnancy [258]. In line with this, a population-based study in Denmark reported that administration of amoxicillin during pregnancy led to higher infant birth weight [158]. Similarly, a prevalence study among Danish schoolchildren aged 7-16 years (n=9666) showed an association between prenatal antimicrobial exposure and overweight/obesity among boys and girls, with a greater risk in boys (adjusted prevalence ratios [aPRs] in boys for overweight: 1.37 and obesity: 1.29) [259].

Evidence from animal studies implicates disruption of the developing gut microbiota in triggering increased obesity risk with early-life antibiotic exposure [130,204]. The inherent instability in the developing gut microbiota creates an ecosystem that is vulnerable to disruption with antibiotics [253]. In particular, early life colonizers such as *Bifidobacterium* that represent 90% of bacterial species in breast fed infants, are highly susceptible to the majority of clinically relevant antibiotics such as penicillin, cephalosporins, macrolides, amoxicillin and clavulanate [148].

Despite mounting evidence from several large studies, human evidence for increased obesity risk due to early life antibiotic exposure remains inconclusive as three recent studies did not detect a significant association between the two [159–161]. Our objective was to analyze data from a community-based prospective pregnancy cohort in Calgary, Canada (All Our Families pregnancy cohort [AOF]) [260–262] to examine the novel question of whether antibiotic exposure during birth (delivery/intrapartum/IAP) is associated with infant body mass index *z* scores (BMI_z) in the first, second and third year of life. In addition, we also examined whether antibiotic use during pregnancy increases infant body mass index *z* scores (BMI_z) in the first, second and third year of life.

5.3 Methods

5.3.1 Study Design and Population

The AOF study (formerly known as the All Our Babies (AOB) study) is a longitudinal prospective pregnancy cohort that recruited 3387 women from community based clinics between May 2008 and December 2010 [263]. Women were eligible for the study if they were at least 18 years of age, less than 25 weeks of gestation, had a

singleton pregnancy, were receiving prenatal care in Calgary and were able to complete the questionnaires in English [263]. Exclusion criteria included planning to move out of Calgary during their pregnancy, carrying multiples at the time of the enrolment, having any of the pre-existing medical conditions (Type 1 or Type 2 diabetes, high blood pressure; autoimmune disorders: lupus, rheumatoid arthritis, Sjogren's syndrome; kidney disease, chronic renal disease, nephritis, nephropathy, dialysis; a heart problem that was repaired by surgery; or chronic infection: hepatitis, HIV). At the time of enrolment participants provided written and oral consent.

Active withdrawals from the study cited loss of interest, lack of time or reasons related to discomfort with blood collections or linkage to medical records [260]. Passive withdrawals occurred due to geographical moves, lack of follow-up, baby loss or unknown reasons [260]. Out of n=4011 pregnant women eligible for recruitment, there were n=2969 recruited for the AOB study. The sample size calculation for the original All Our Babies cohort study was based on planned microarray analysis [263]. Data for the current study was drawn from only women who had intrapartum antibiotic exposure documented in their obstetrical and birth record (n=1303; objective 1) or self-reported their antibiotic usage during pregnancy (n=1943; objective 2). The resulting sample size is adequate to describe differences between groups with respect to antibiotic exposure (present or absent) and obesity risk. The Child Health Research Office and the Conjoint Health Research Ethics Board (CHREB) at the University of Calgary approved the original AOF cohort study (Ethics ID 20821 and 22821). This antibiotic sub-study was approved by the CHREB (REB15-2192).

5.3.2 Questionnaire Data Collection

Questionnaires were developed to gather AOB cohort outcomes addressing demographics, pregnancy history, health service utilization, nutrition and exercise practices, breastfeeding experiences, lifestyle and life history and several other outcomes, which are not relevant for the purpose of the present analysis [261]. The questionnaires were designed using the Cardiff Teleform software suite (Cardiff Teleform, Version 10.1, 2007) and pilot tested before the AOB study [261]. There were 6 questionnaires mailed out and answered at: (Q1) 24 weeks of gestation, (Q2) 34-36 weeks of gestation, (Q3) 4 months post-partum, (Q4) 1 year post-partum, (Q5) 2 years post-partum, (Q6) 3 years

post-partum [261]. Obstetrical/birth record data was acquired from a provincial medical database which contains birth outcome information from all births in Alberta, Canada [261].

5.3.3 Antibiotic exposure

Data for the current study was drawn only from women who had intrapartum antibiotic exposure documented in their obstetrical/birth record (n=1303; objective 1, documented as "yes", "no" or was missing) or self-reported their antibiotic usage during pregnancy in the 34-36 week questionnaire (n=1943; objective 2). It was an open-ended question asking whether mothers received any prescription medications during pregnancy. Maternal responses were coded as: 0 – did not receive antibiotics during pregnancy and 1 – did receive antibiotics during pregnancy. Since this was a sub-analysis of the pre-existing data, we could not investigate dosage(s), timing and types of antibiotics prescribed during pregnancy because the data was not available.

5.3.4 Infant BMI

Infant/child body weight and height at the first, second and third year of life were acquired from questionnaires. Age- and sex-specific BMI-for-age *z* scores were calculated according to the 2006 World Health Organization standards using their "igrowup" package for R (Shinny Apps) [264,265].

5.3.5 Covariates

Maternal age, ethnicity, exercise, weight gain, milk/fruit/grain consumption, antifungal/antiviral use during pregnancy, BMI at the end of pregnancy, number of previous pregnancies, smoking (during pregnancy/after birth) and breastfeeding were ascertained by self-reported questionnaires during pregnancy/post-partum. Weight gain during pregnancy was determined by pre-pregnancy weight and weight at the end of pregnancy (Q2). Mothers were defined as smokers if they reported smoking at any time during pregnancy or after birth. Paternal age and ethnicity as well as infants' introduction to solid foods and infant sex were also acquired from questionnaires. From the electronic health records we obtained information on the presence of diabetes during pregnancy (preexisting or gestational), type of delivery, term status and infant's gestational age. Small for gestational age was defined as birth weight below the 10^{th} percentile of sex-

specific birth weight and large for gestational age was birth weight above the 90th percentile of sex-specific birth weight.

5.3.6 Statistical Analysis

The association between covariates and exposure to antibiotics during pregnancy and IAP was examined using χ^2squared tests (Table 5.1). The univariate association between each potential covariate and BMI *z* scores at year 1, 2, and 3 was also examined. We used a linear mixed model repeated measures analysis (STATA v14 Mixed) to examine the association between mothers' exposure to IAP and/or antibiotics during pregnancy and infant's BMI *z* scores at year 1, 2 and 3. Antibiotic exposure during pregnancy and IAP as well as potential confounders were included as fixed effects. Two interaction terms (antibiotics during pregnancy × year and IAP × year) were included in initial models. Variables were explored as potential confounders in the final model if they were associated with either exposure or associated with BMI *z* scores (Table 5.3). Backward regression was used to determine the most parsimonious model. In all mixed model analyses, each participant was treated as a random variable. Both unadjusted and adjusted models are presented. Statistical significance was set at $P<0.05$. To assess the impact of missing data, we used two different sensitivity analysis approaches. The first approach replaced missing exposure and covariate values with a level in the model and the second model was multiple chained imputation with 10 iterations.

5.4 Results

Among 1972 women included in the analysis, 71.0% were between 25-35 years old, white (81.9%) and exercised 0-2 times a week (55.5%). Similarly, the majority of fathers were between 25-35 years old (50.2%) and white (79.8%). Mothers were non-smokers (90.2%), had overweight (43.8%) or obesity (31.0%) at the end of pregnancy and gained excessive weight during pregnancy (42.0%). Maternal dietary habits during pregnancy consisted of 2-4 servings of milk (71.6%), 2-4 servings of fruit (52.1%) and 1 serving of grains per day (77.1%). Maternal income was >$60,000 CAD per year during pregnancy (81.9%), most had been pregnant previously (1-3 previous pregnancies, 57.8%), had no gestational diabetes (82.8%) and did not take antiviral/antifungal medication during pregnancy (97.1%) (Table 5.1). Of 1303 women with available IAP

data from the obstetrical/birth record, 441 received antibiotic and 862 did not receive antibiotic during birth. Of 1943 with available data on antibiotic use during pregnancy, a total of 187 indicated they had taken antibiotics.

Newborns (51.1% boys and 47.1% girls) were delivered vaginally (93.5%), at term (91.3%) and were normal size for gestational age (82.8%). The majority of the newborns were breastfed (58.6%) and introduced to solid foods between 4-6 months (48.3%), however there was a high degree of missing data for those two outcomes (39.2% and 41.1%, respectively, Table 5.2). The majority of children were active 0 - 3 times per week when 2 and 3 years old. Most children (63.1%) had no infection(s) in the first three years of life, but those who did, reported having between 1-2 infections in the first three years of life (24.0%) (Table 5.1).

The distribution of covariates across antibiotic use is shown in Table 5.1. The number of previous pregnancies was associated with intrapartum antibiotic exposure, whereas fruit consumption, gestational diabetes, gestational age and sex of the baby were associated with antibiotic exposure during pregnancy. Term status (term/preterm) was associated with both antibiotic exposure types and a negative association was found between preterm status and BMI *z* score at 2 years of age.

Table 5.1 Parental and child characteristics according to antibiotic exposure

	Intrapartum antibiotics (n=1303)			**Antibiotic use during pregnancy (n=1943)**			**All women combined (n=1972)**
	No	Yes	P Value	No	Yes	P Value	
Maternal age, n (%)			0.139			0.060	
≤24	51 (5.9)	39 (8.8)		109 (6.2)	20 (10.7)		131 (6.6)
25 to 35	623 (72.3)	320 (72.6)		1258 (71.6)	123 (65.8)		1401 (71.0)
>35	169 (19.5)	71 (16.1)		340 (19.4)	41 (21.9)		388 (19.7)
Missing	20 (2.3)	11 (2.5)		49 (2.8)	3 (1.6)		52 (2.6)
Maternal ethnicity			0.368			0.140	
White	698 (81.0)	343 (77.8)		1447 (82.4)	146 (78.1)		1615 (81.9)
Other	163 (18.9)	97 (22.0)		298 (17.0)	41 (21.9)		346 (17.5)
Missing	1 (0.1)	1 (0.2)		11 (0.6)	0 (0)		11 (0.6)
Paternal age, n (%)			0.468			0.077	
≤24	32 (3.7)	10 (2.3)		52 (3.0)	2 (1.1)		56 (2.8)
25 to 35	438 (50.8)	231 (52.4)		874 (49.7)	106 (56.7)		990 (50.2)
>35	251 (29.1)	134 (30.3)		505 (28.8)	55 (29.4)		570 (28.9)
Missing	141 (16.4)	66 (15.0)		325 (18.5)	24 (12.8)		356 (18.1)
Paternal ethnicity			0.610			0.397	
White	683 (79.2)	339 (76.8)		1427 (81.3)	145 (77.5)		1574 (79.8)
Other	155 (18.0)	89 (20.2)		312 (17.7)	39 (20.9)		351 (17.8)
Missing	24 (2.8)	13 (3.0)		17 (1.0)	3 (1.6)		47 (2.4)

Maternal exercise during pregnancy			0.112		0.810	
≥6 x/week	73 (8.5)	29 (6.6)	134 (7.6)	18 (9.6)		153 (7.8)
3-5 x/week	309 (35.8)	156 (35.4)	626 (35.7)	64 (34.2)		704 (35.7)
0-2 x/week	474 (55.0)	247 (56.0)	977 (55.6)	103 (55.1)		1094 (55.4)
Missing	6 (0.7)	9 (2.0)	19 (1.1)	2 (1.1)		21 (1.1)
Maternal smoker (during pregnancy or after birth)			0.091		0.131	
Non-Smoker	789 (91.5)	390 (88.4)	1575 (89.7)	176 (94.1)		1778 (90.2)
Smoker	71 (8.3)	51 (11.6)	172 (9.8)	11 (5.9)		183 (9.2)
Missing	2 (0.2)	0 (0)	9 (0.5)	0 (0)		11 (0.6)
Maternal weight gain during pregnancy			0.393		0.869	
Adequate	305 (35.4)	142 (32.2)	606 (34.5)	63 (33.7)		676 (34.3)
Inadequate	140 (16.2)	68 (15.4)	276 (15.8)	33 (17.6)		316 (16.0)
Excessive	364 (42.2)	195 (44.2)	738 (42.0)	75 (40.1)		828 (42.0)
Missing	53 (6.2)	36 (8.2)	136 (7.7)	16 (8.6)		152 (7.7)
Maternal BMI end of pregnancy			0.119		0.577	
Normal	181 (21.00)	73 (16.6)	337 (19.2)	38 (20.3)		380 (19.3)
Underweight	0 (0)	1 (0.2)	4 (0.2)	0 (0)		4 (0.2)
Overweight	389 (45.1)	194 (44.0)	763 (43.5)	88 (47.1)		863 (43.8)
Obesity	253 (29.4)	150 (34.0)	545 (31.0)	54 (28.9)		611 (31.0)
Missing	39 (4.5)	23 (5.2)	107 (6.1)	7 (3.7)		114 (5.7)

Maternal milk consumption during pregnancy, servings			0.235		0.323	
≥ 5 per day	44 (5.1)	24 (5.4)		92 (5.2)	10 (5.4)	102 (5.2)
2-4 per day	615 (71.4)	323 (73.3)		1254 (71.4)	133 (71.1)	1412 (71.6)
1 per day	175 (20.2)	89 (20.2)		357 (20.3)	37 (19.8)	398 (20.1)
None	25 (2.9)	4 (0.9)		34 (1.9)	7 (3.7)	41 (2.1)
Missing	3 (0.4)	1 (0.2)		19 (1.2)	0 (0)	19 (1.0)
Maternal fruit consumption during pregnancy, servings			0.998		0.009	
≥ 5 per day	51 (5.9)	26 (5.9)		112 (6.4)	14 (7.5)	127 (6.4)
2-4 per day	441 (51.2)	228 (51.7)		916 (52.2)	95 (50.8)	1027 (52.1)
1 per day	365 (42.3)	184 (41.7)		707 (40.3)	75 (40.1)	794 (40.3)
None	3 (0.4)	2 (0.5)		3 (0.2)	3 (1.6)	6 (0.3)
Missing	2 (0.2)	1 (0.2)		18 (1.0)	0 (0)	18 (0.9)
Maternal grain consumption during pregnancy, servings			0.586		0.182	
≥ 5 per day	12 (1.4)	3 (0.7)		27 (1.5)	1 (0.5)	28 (1.4)
2-4 per day	173 (20.1)	79 (17.9)		360 (20.5)	34 (18.2)	396 (20.1)
1 per day	670 (77.6)	356 (80.7)		1343 (76.6)	150 (80.2)	1520 (77.1)
None	4 (0.5)	1 (0.2)		6 (0.3)	2 (1.1)	8 (0.4)
Missing	3 (0.4)	2 (0.5)		20 (1.1)	0 (0)	20 (1.0)
Maternal antifungal or antiviral use during pregnancy			0.433		0.049	
	821 (95.2)	415 (94.1)		1701 (96.9)	187 (100)	1888 (97.1)
No	12 (1.4)	5 (1.1)		23 (1.3)	0 (0)	23 (1.1)
Yes	29 (3.4)	21 (4.8)		32 (1.8)	0 (0)	34 (1.8)
Missing						

Number of previous pregnancies			<0.001		0.103	
0	282 (32.7)	203 (46.0)		657 (37.4)	53 (28.3)	723 (36.7)
1-3	540 (62.7)	218 (49.4)		1004 (57.2)	121 (64.7)	1140 (57.8)
4-8	31 (3.6)	17 (3.9)		72 (4.1)	10 (5.4)	83 (4.2)
Missing	9 (1.0)	3 (0.7)		23 (1.3)	3 (1.6)	26 (1.3)
Income during pregnancy (per anum)			0.132		0.101	
>=60,000	707 (82.0)	347 (78.7)		1459 (83.1)	153 (81.8)	1614 (81.9)
<60,000	121 (14.1)	80 (18.1)		225 (12.8)	31 (16.6)	282 (14.3)
Missing	34 (3.9)	14 (3.2)		72 (4.1)	3 (1.6)	76 (3.8)
Maternal gestational diabetes			0.708		0.034	
No	825 (95.7)	424 (96.2)		1697 (96.6)	175 (93.6)	1900 (96.4)
Yes	37 (4.3)	17 (3.8)		59 (3.4)	12 (6.4)	72 (3.6)
Infant Sex			0.446		<0.001	
Girl	411 (47.7)	196 (44.4)		824 (46.9)	90 (48.1)	929 (47.1)
Boy	432 (50.1)	237 (53.8)		909 (51.8)	86 (46.0)	1008 (51.1)
Missing	19 (2.2)	8 (1.8)		23 (1.3)	11 (5.9)	35 (1.8)
Term Status			<0.001		0.004	
Term	808 (93.7)	366 (83.0)		1610 (91.7)	163 (87.2)	1800 (91.3)
Preterm	32 (3.7)	61 (13.8)		111 (6.3)	13 (7.0)	125 (6.3)
Missing	22 (2.6)	14 (3.2)		35 (2.0)	11 (5.8)	47 (2.4)
Infant size based on gestational age (GA)			0.601		<0.001	
Normal	639 (74.1)	325 (73.7)		1327 (75.6)	122 (65.2)	1633 (82.8)
Small for GA	80 (9.3)	48 (10.9)		173 (9.9)	15 (8.0)	196 (9.9)
Large for GA	68 (7.9)	37 (8.4)		150 (8.5)	15 (8.1)	143 (7.3)
Missing	75 (8.7)	31 (7.0)		106 (6.0)	35 (18.7)	0 (0)

Mode of delivery			<0.001		0.978	
Vaginal	834 (96.8)	374 (84.8)		1649 (93.9)	175 (93.6)	1843 (93.5)
C-section	28 (3.2)	63 (14.3)		97 (5.5)	11 (5.9)	117 (5.9)
Missing	0 (0)	4 (0.9)		10 (0.6)	1 (0.5)	12 (0.6)
Breastfeeding			0.402		0.372	
Yes	524 (60.8)	279 (63.2)		1025 (58.4)	115 (61.5)	1156 (58.6)
No	20 (2.3)	6 (1.4)		37 (2.1)	6 (3.2)	43 (2.2)
Missing	318 (36.9)	156 (35.4)		694 (39.5)	66 (35.3)	773 (39.2)
Introduction to solid foods			0.747		0.178	
0 to<4 months	33 (3.8)	17 (3.9)		52 (3.0)	5 (2.7)	60 (3.0)
4 to <6 months	424 (49.2)	227 (51.5)		838 (47.7)	102 (54.6)	953 (48.3)
6 to <8 months	54 (6.3)	31 (7.0)		112 (6.4)	5 (2.6)	117 (5.9)
>8 months	16 (1.9)	5 (1.1)		28 (1.6)	4 (2.1)	32 (1.6)
Missing	335 (38.8)	161 (36.5)		726 (41.3)	71 (38.0)	810 (41.2)
Physical Activity at 2 years (times/week)			0.014		0.841	
≥5	204 (23.7)	112 (25.4)		389 (22.2)	40 (21.4)	196 (12.7)
3<5	174 (20.2)	115 (26.1)		382 (21.8)	46 (24.6)	241 (15.6)
0-<3	213 (24.7)	80 (18.1)		390 (22.2)	41 (21.9)	433 (28.2)
Missing	271 (31.4)	134 (30.4)		595 (33.8)	60 (32.1)	670 (43.5)
Physical Activity at 3 years (times/week)			0.299		0.690	
≥7	57 (6.6)	37 (8.4)		125 (7.1)	11 (5.9)	139 (7.1)
5-<7	120 (13.9)	64 (14.5)		252 (14.4)	29 (15.5)	285 (14.5)
3<5	276 (32.0)	152 (34.5)		569 (32.3	67 (35.8)	647 (32.8)
1-<3	380 (44.1)	177 (40.1)		749 (42.7)	77 (41.2)	836 (42.3)
<1	29 (3.4)	10 (2.3)		59 (3.4)	3 (1.6)	63 (3.2)
Missing	0 (0)	1 (0.2)		2 (0.1)	0 (0)	2 (0.1)

Ear Infections			0.910		0.553	
No	547 (63.5)	283 (64.2)		1107 (63.0)	117 (62.6)	1244 (63.1)
Yes	305 (35.4)	152 (34.5)		628 (35.8)	66 (35.3)	702 (35.6)
Missing	10 (1.1)	6 (1.3)		21 (1.2)	4 (2.1)	26 (1.3)
Number of Ear Infections in the first 3 years of life			0.950		0.428	
0	547 (63.5)	283 (64.2)		1107 (63.0)	117 (62.6)	1244 (63.1)
1-2	205 (23.8)	97 (22.0)		420 (23.9)	48 (25.7)	474 (24.0)
3-4	58 (6.7)	33 (7.5)		125 (7.1)	11 (5.9)	136 (6.9)
≥5	41 (4.8)	22 (5.0)		82 (4.7)	6 (3.2)	90 (4.6)
Missing	11 (1.2)	6 (1.3)		22 (1.3)	5 (2.6)	28 (1.4)

Associations between potential confounders and antibiotic use were examined using χ^2squared tests. *P<0.05

The association between BMI *z* score and antibiotic exposure is presented in Table 5.2 and Fig. 5.1A-B. An association between intrapartum antibiotic exposure and BMI *z* score at year 1 (p=0.013) and year 2 (p=0.018), but not year 3 (p=0.491) of life was observed (Fig. 5.1A). The highest BMI *z* score was observed among 1 year old children born to mothers given intrapartum antibiotics (mean [SE]; 1.317 [0.194] vs. 0.755 [0.130] for exposed to IAP vs. not exposed; estimated mean difference 0.555; 95% CI, 0.116 – 0.995). Similarly, higher BMI *z* scores were observed among 2 year olds when their mothers received intrapartum antibiotics (mean [SE]; 0.850 [0.107] vs. 0.586 [0.070] for exposed to IAP vs. not exposed; estimated mean difference 0.295; 95% CI, 0.050 – 0.540). After adjusting for additional covariates (maternal antibiotic use during pregnancy, maternal weight gain during pregnancy, maternal age, diet, number of previous pregnancies, gestational diabetes, term status, infant size based on gestational age, sex of the baby and infant's number of ear infections; Table 5.3), an association was no longer present at 2 years of age (estimated mean difference 0.231 units; 95%CI, -0.038 – 0.499). However, in the final adjusted linear mixed model (Table 5.2, Fig. 5.2), maternal intrapartum antibiotic exposure remained significantly associated with BMI *z* score at 1 year of age (β [95% CI]; 0.567 unit increase [0.094 – 1.04] for exposed to IAP vs. not exposed). To assess the impact of missing data, we used two different approaches. First, we conducted sensitivity analysis where missing data was replaced with a level in the model in order to address the concern of the data not missing completely at random. We confirmed the results we found in the final adjusted linear mixed model showing an association between intrapartum antibiotic exposure and BMI *z* scores at 1 year of age (β [95% CI]; 0.584 unit increase [0.141 – 1.026] for exposed to IAP vs. not exposed). Second, we performed multiple chain imputation sensitivity analysis and the final adjusted model no longer showed a significant interaction between IAP and year (p=0.059). No association was found between antibiotic exposure during pregnancy and BMI *z* scores (Fig. 5.1B).

Table 5.2 Maternal antibiotic exposure (during birth and pregnancy) and child BMI *z* score at 1, 2, 3 years of age

	Mean (SE)	95% CI	Estimated mean difference (95% CI) from unadjusted model	Estimated mean difference (95% CI) from adjusted model
	Child BMI z score at 1 Year			
During birth (IAP) (n=753)				
NO	0.755 (0.130)	0.500 - 1.010	(ref)	(ref)
YES	1.317 (0.194)*	0.936 - 1.699*	0.555 (0.116 - 0.995)*	0.567 (0.094 – 1.040)*
During pregnancy (n=1074)				
NO	0.881 (0.095)	0.695 - 1.067	(ref)	
YES	1.124 (0.297)	0.535 - 1.713	0.247 (-0.339 - 0.832)	
	Child BMI *z* score at 2 Years			
During birth (IAP) (n=837)				
NO	0.586 (0.070)	0.449 - 0.724	(ref)	(ref)
YES	0.850 (0.107)*	0.639 - 1.061*	0.295 (0.050 - 0.540)*	0.231 (-0.038 – 0.499)
During pregnancy (n=1200)				
NO	0.678 (0.054)	0.573 - 0.783	(ref)	
YES	0.901 (0.174)	0.557 - 1.246	0.180 (-0.160 - 0.520)	

	Child BMI *z* score at 3 Years			
During birth (IAP) (n=1248)				
NO	0.217 (0.048)	0.122 - 0.311	(ref)	(ref)
YES	0.266 (0.057)	0.154 - 0.378	0.054 (-0.100 - 0.208)	0.046 (-0.122 – 0.213)
During pregnancy (n=1858)				
NO	0.211 (0.032)	0.149 - 0.273	(ref)	
YES	0.270 (0.103)	0.065 - 0.474	0.062 (-0.144 - 0.268)	

Abbreviations: BMI, body mass index

Adjusted model controls for antibiotic exposure during pregnancy, maternal weight gain during pregnancy, maternal age, diet, number of previous pregnancies, gestational diabetes, term status, infant size based on gestational age, sex of the baby and infant's number of ear infections in the first three years of life. *P<0.05.

Table 5.3 Associations between confounders and BMI *z* score (Full Model)

	β (95% CI)
Intrapartum antibiotic exposure	
NO	[ref]
YES	0.567 (0.094-1.040)*
Year	
1	[ref]
2	-0.080 (-0.382-0.222)
3	-0.417 (-0.693 –[-0.141])
Intrapartum antibiotic exposure × year	
NO exposure, year 1	[ref]
YES exposure, year 2	-0.336 (-0.850 – 0.178)
YES exposure, year 3	-0.521 (-0.991 – [-0.300])*
Antibiotic exposure during pregnancy	
NO	[ref]
YES	0.054 (-0.192 – 0.300)
Maternal age (years)	
≤24	[ref]
25 to 35	-0.084 (-0.382 – 0.213)
>35	-0.151 (-0.485 – 0.182)

Maternal weight gain during pregnancy	
Adequate	[ref]
Inadequate	-0.115 (-0.329 – 0.099)
Excessive	0.268 (0.109 – 0.428)*
Maternal fruit consumption during pregnancy, servings	
≥ 5 per day	[ref]
2-4 per day	0.064 (-0.252 – 0.380)
1 per day	0.1 (-0.227 – 0.428)
None	0.922 (-0.299 – 2.144)
Maternal grain consumption during pregnancy, servings	
>= 5 per day	[ref]
2-4 per day	0.371 (-0.272 – 1.014)
1 per day	0.552 (-0.078 – 1.182)
None	0.894 (-0.632 – 2.420)
Number of previous pregnancies	
0	[ref]
1-3	-0.031 (-0.187 – 0.125)
4-8	0.278 (-0.152 – 0.709)
Term Status	
Term	[ref]
Preterm	-0.182 (-0.496 – 0.133)

Infant size based on gestational age (GA)	
Normal	[ref]
Small for GA	-0.382 (-0.623 –[-0.141])*
Large for GA	0.135 (-0.124 – 0.393)
Maternal gestational diabetes	
NO	[ref]
YES	-0.234 (-0.606 – 0.138)
Infant Sex	
Girl	[ref]
Boy	0.129 (-0.017 – 0.276)
Number of Ear Infections in the first 3 years of life	
0	[ref]
1-2	0.050 (-0.123 – 0.223)
3-4	-0.047 (-0.330 – 0.236)
≥5	0.656 (0.305 – 1.007)*

Univariate association between confounders and the outcome (BMI *z* score) were examined using linear regression analysis. *P<0.05

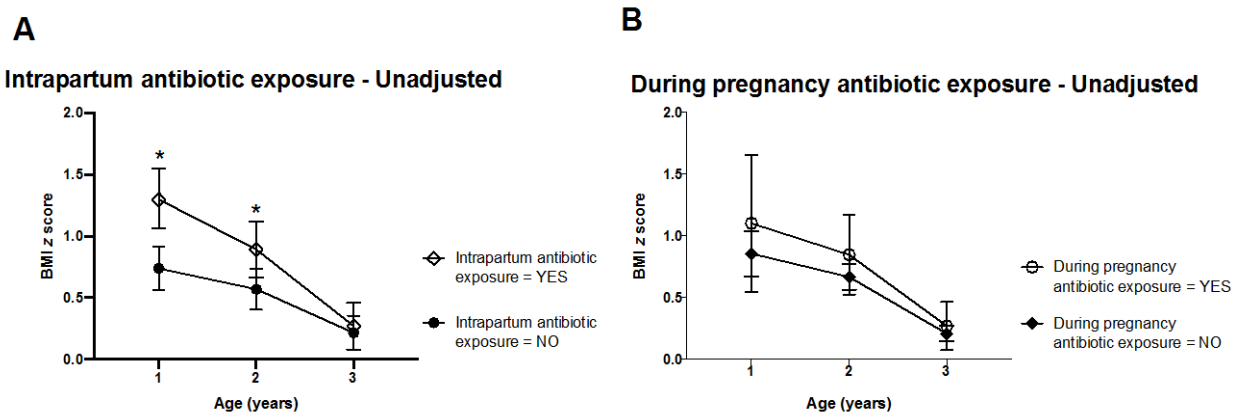

Figure 5.1 Maternal antibiotic exposure and child BMI *z* score at 1, 2, 3 years of age (Unadjusted Model)

Age- and sex-specific estimated BMI-for-age *z* scores were calculated according to the 2006 World Health Organization standards. Error bars indicate 95% CI. (A) Intrapartum antibiotic exposure and child BMI *z* score at 1, 2, 3 years of age, (B) Antibiotic exposure during pregnancy and child BMI *z* score at 1, 2, 3 years of age. BMI, body mass index, *P<0.05

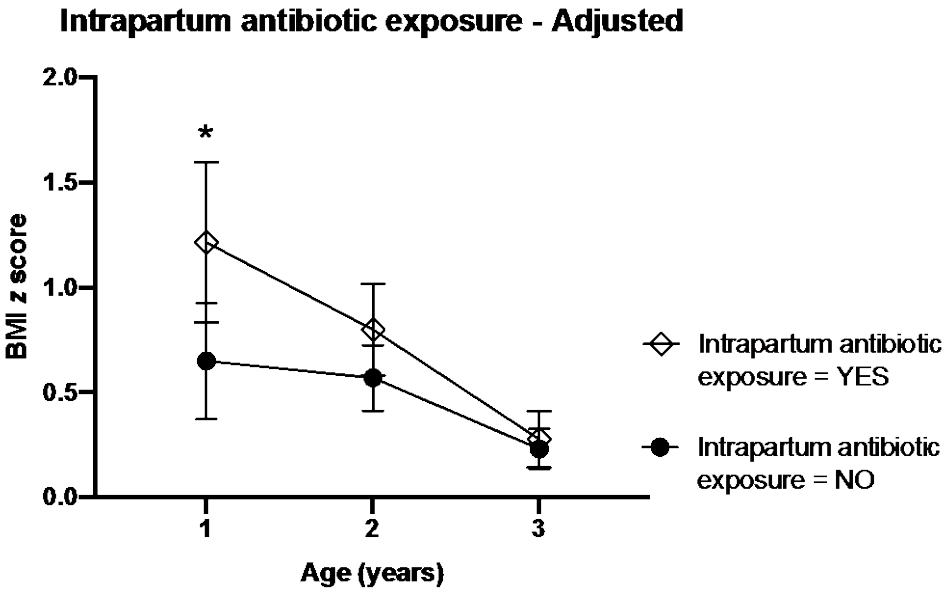

Figure 5.2 Maternal intrapartum antibiotic exposure and child BMI *z* score at 1, 2, 3 years of age (Final Adjusted Linear Mixed Model)

Age- and sex-specific BMI-for-age *z* scores were calculated according to the 2006 World Health Organization standards. Error bars indicate 95% CI.

Final adjusted linear mixed model includes an interaction term for intrapartum antibiotic exposure and year and controls for antibiotic exposure during pregnancy, maternal weight gain during pregnancy, maternal age, diet, number of previous pregnancies, gestational diabetes, term status, infant size based on gestational age, sex of the baby and infant's number of ear infections in the first three years of life. BMI, body mass index, *P<0.05

5.5 Discussion

Our findings in this AOF cohort study indicate that maternal antibiotic exposure during birth (IAP) is associated with a 0.56 and 0.30 unit increase in child BMI z-score at 1 and 2 year of age, respectively. No comparable associations were found for antibiotic exposure during pregnancy and BMI *z* scores. In the final adjusted linear model, which controlled for known obesity risk factors such as maternal weight gain during pregnancy, size for gestational age, number of infections in the first three years of life and antibiotic exposure during pregnancy, maternal IAP was associated with a 0.57 unit increase in BMI *z* score at 1 year of age compared to no IAP exposure.

Risk for chronic diseases including obesity is influenced by the postnatal environment as well as maternal programming during the gestational period [266]. Animal [130,204,267] and multiple epidemiological human studies [18–20,155,156,165,268] suggest that early life antibiotic exposure is associated with increased adiposity and weight gain later in life. To our knowledge, this is the first human study to investigate the association between intrapartum antibiotic exposure and infant/child BMI *z* score at the early timepoints of 1 and 2 years of age. Of interest is that we saw increased BMI *z* score at 1 year of age with IAP, but the difference was no longer present at 2 and 3 years of age. In line with this finding, no association was found between antibiotic exposure during labor/delivery and BMI *z* score at 3 years of age in a retrospective cohort study of 8793 mother-child dyads in the USA [269]. However, since the study did not investigate BMI *z* score at 1 and 2 years of age, it is not known if an association existed earlier in life. An explanation for a higher

BMI *z* score at 1 year of age following antibiotic exposure can be explained by animal models where this trend is commonly observed [130,204]. In animal studies, growth rates in the first 10 days post-weaning are significantly elevated when mothers are exposed to antibiotics during pregnancy/lactation [130,204]. Although phenotypic differences in the offspring at 10 days post-weaning are still minimal, when animals are exposed to a high-fat diet in adulthood, the full extent of the obesity risk is unmasked and increased fat mass is seen in offspring of mothers treated with antibiotics during pregnancy/lactation [130]. Therefore, despite the differences in BMI *z* score being diminished at 2 and 3 years of age, it is possible that infants exposed to IAP are more susceptible to developing obesity later in life if they were exposed to a Western-style diet high in sugar, fat and processed low fiber foods.

Cox *et al.* (2012) [130] demonstrated that maternal antibiotic exposure increased periweaning (d21-28) growth rates of rodent offspring, with the difference being minimized quickly thereafter, which is similar to what we observed with 2 and 3 year old data. However, when mice were exposed to a high fat, high sugar diet later in life, antibiotic-exposed offspring quickly became heavier than controls. In line with this, a meta-analysis with 47,661 participants from 10 cohort studies showed that increased infant weight gain in the first year of life was a risk factor for obesity in childhood (6-14 years) and adulthood (ages 17-66 years) [8]. Therefore a longer duration follow up of this study would be necessary to investigate whether increased infant growth rates seen after IAP contribute to obesity in the AOF cohort later in life especially because early life microbiota programming alterations often require a metabolic insult (e.g. Western-style diet) to reveal an obese phenotype [130]. Nevertheless, this increase in BMI *z* score is clinically relevant as a modest 0.1 unit increase in BMI *z* score has been associated with worsening of cardiovascular risk factors such as C-peptide and total/HDL cholesterol [270]. On the other hand, a small decrease of 0.1 unit in BMI *z* score improved insulin, insulin resistance, total and LDL cholesterol and total/HDL cholesterol [270].

Our study showed that maternal antibiotic exposure is associated with maternal weight gain during pregnancy, maternal age, diet, term status, number of previous pregnancies, gestational diabetes, infant size based on gestational age, sex of the baby and infant's number of ear infections [159,271], which are known risk factors for obesity.

Controlling for these factors, did not explain the association between IAP exposure and BMI *z* score at 1 year of age, therefore suggesting an independent effect of IAP on infant body weight.

Furthermore, experimental studies in animals show that the most critical period to affect obesity risk with antibiotics is at the time of birth [130]. This window of vulnerability is due to the mammalian newborn harbouring a simple gut microbial community with low diversity, low stability and low resilience (the ability to recover after an insult) [10]. Disrupting microbiota development and reducing early-life protective bacterial populations leads to changes in metabolic and/or immunologic development with increased adiposity emerging early- to mid-adulthood [130]. Even though intrapartum antibiotic exposure is considered to be safe in humans, disturbances in non-target populations in maternal gut microbiota with IAP and lack of the vertical transmission of early-life colonizers (i.e. *Lactobacillus*) [13] to the infant during birth pose a risk for diseases later in life [14]. Furthermore, intrapartum antibiotics directly target the baby through placental fetal/neonatal bloodstream, further interrupting the microbiota seeding of the baby at birth [14]. In animal studies [130,204] penicillins have the strongest impact on obesity risk, which is a common antibiotic used during IAP treatment [14,272]. In one study, IAP administration significantly altered early gut microbial profiles in infants, most notably a decrease of 7.2% in the relative abundance of *Bifidobacterium* was seen with every hour of IAP administration [14]. Several studies have reported a correlation between low levels of *Bifidobacterium* and obesity [15–17]. It is possible that AOF infants born to mothers exposed to IAP had lower *Bifidobacterium* and were therefore at higher risk of obesity especially as *Bifidobacterium* are sensitive to antibiotics [148].

In contrast to previous studies [12,158,257] where maternal exposure during pregnancy was associated with increased BMI *z* score in children, we were unable to detect an association. Nonetheless, our findings are in line with the study by Poulsen *et al.* (2017) [269] where no association was found between prenatal antibiotic exposure and child BMI at 3 years of age. It is possible to explain our findings with the key limitation of our study: self-reported antibiotic use during pregnancy whereas IAP was obtained from the obstetrical/birth record. We did not differentiate between broad and narrow spectrum antibiotics, the dose administered and the timing of administration during pregnancy, all

factors that have an impact on obesity risk associated with antibiotic exposure [257]. Previous research showed that second/third trimester multiple broad-spectrum antibiotic exposures have the strongest impact on obesity [20,155,257]. The second and third trimester of pregnancy is when the fetal intestine is developing [273] and since antibiotics can alter maternal microbiota [274] and enter fetal circulation through the placenta [275], it is possible that a combination of these factors impairs intestinal development during late pregnancy and seeding of the infant's intestinal microbiota [12]. It is also possible that we did not find an association between maternal antibiotic use during pregnancy and infant BMI *z* score because of a single antibiotic exposure with a narrow spectrum of activity or that antibiotics were taken during early pregnancy and outside of the typically associated second and third trimester.

The major strengths of our study are that we acquired data about antibiotic exposure during birth (IAP) through medical database and not self-reported questionnaires and we were able to control for several risk factors contributing to obesity. On the other hand, limitations include possible errors with self-reported maternal antibiotic exposure during pregnancy and lack of information about the dose/timing/type of administration and as well as missing data (covariates and main outcome) at 1 and 2 years of age. Furthermore, the lack of more detailed information for certain variables such as breastfeeding and smoking might further limit our results because we were limited categorical reporting (i.e. Yes/No) and duration and timing of these factors can affect obesity risk.

5.6 Conclusions

To our knowledge, our results provide the first human evidence that maternal intrapartum antibiotic exposure increases child BMI *z* score at 1 year of age. Given the high prevalence of childhood obesity and high prescription rates of intrapartum antibiotics, future studies should investigate the mechanisms underlying these findings. By understanding why there is an increase in BMI *z* score at 1 year of age associated with intrapartum antibiotics, future intervention studies could be undertaken to prevent the risk and consequently reduce the burden of obesity.

5.7 Acknowledgments

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5.8 Author Contributions

Teja Klancic cleaned the data and drafted the paper. Amanda Black performed statistical analysis. All authors critically reviewed the manuscript for important intellectual content. Raylene A. Reimer had primary responsibility for final content.

CHAPTER SIX: CONCLUSION

6.1 Introduction

Antibiotics were first prescribed during World War II to treat serious infections. Its' use revolutionized medicine and saved millions of lives helping in part to increase life expectancy by more than 40% in the USA since the 1920s.[276] Nevertheless, only recently has the extent of the negative side effects of antibiotic use such as antibiotic resistance, immune disorders and metabolic impairments been more fully described.[276] Despite warnings regarding antibiotic overuse, they remain heavily overprescribed as well as available to buy without prescription and used as growth-promoting agents in livestock in many countries.[277]

Included in the negative side effects of antibiotic use is an increased risk of obesity, a complex disease with multiple contributing factors. One of the most intensely studied factors affecting health and disease during the past decade has been the gut microbiota. The gut microbiota, via energy extraction, inflammation and other actions is now recognized as an important player in the pathogenesis of obesity. Dysbiosis or an imbalance in the microbial community can initiate a cascade of metabolic disturbances in the host. Early life is a particularly important period for the development of the gut microbiota and perturbations such as with antibiotic exposure can have long-lasting consequences for host health. In early life and throughout the life span, diet is one of the most important factors that shape the gut microbiota. While diets high in fat and sugar have been shown to contribute to dysbiosis and disease, dietary fiber is recognized as an important fermentative fuel for the gut microbiota and results in the production of SCFAs that can act as signaling molecules in the host. One particular type of fiber, prebiotic fiber contributes to changes in the gut microbiota, most notable of which is an increase in the abundance of *Bifidobacterium*.[35]

The goal of this dissertation was to investigate the potential of co-administering prebiotics with antibiotics in early life to reduce obesity risk in maternal and direct pup exposure models. Furthermore, obesity risk in children exposed to antibiotics via maternal antibiotic use during pregnancy or during birth was assessed with data from the AOF cohort. Three major studies were carried out to answer those questions. The first

animal study examined the impact of indirect (maternal) antibiotic exposure with prebiotic co-administration on obesity risk in the offspring. The second study employed a direct antibiotic/prebiotic exposure in young rat pups and assessed obesity risk and other metabolic parameters. The final study analyzed the human birth cohort data from the AOF study and explored the association between maternal antibiotic exposure and infant BMI *z*-scores.

6.2 General discussion

The major research findings from the three studies are:

1. Low dose penicillin use during pregnancy/lactation impairs normal postpartum weight loss in dams. Co-administering oligofructose with penicillin prevented obesity risk in dams and their offspring, likely in part through maternal and offspring microbiota alterations.
2. Therapeutic doses of azithromycin administered to rats pups increase body weight and impair insulin production and insulin sensitivity but the effects were reversed with oligofructose co-administration in a sex-specific manner.
3. Exposure to antibiotics during birth may contribute to the development of child overweight/obesity at 1 year of age.

6.2.1 Early life antibiotic exposure increases body weight, impairs metabolism and gut microbiota composition of the dams and their offspring

Based on the evidence from previous studies in humans and mice and the growth-promoting effects of antibiotics in production animals[3], we decided to assess the potential to induce obesity in rats with a beta-lactam (penicillin) and macrolide (azithromycin) as these are two of the most commonly used antibiotics early in life[278]. While it was previously reported that low doses of penicillin administered to mice during pregnancy/lactation promote growth in their offspring[130], we are the first to report the impact of the antibiotics on the metabolic health of the dams including reduced postpartum weight loss following the same antibiotic exposure as used previously in mice by Cox *et al*[130]. Low doses of antibiotics are commonly used in livestock for growth promotion[276], therefore it is not surprising that a robust obese phenotype was established in our first animal study. Antibiotic dams remained significantly heavier post-partum

with elevated fat mass, leptin and hepatic triglycerides. Their offspring showed accelerated early life post weaning growth rates and increased body weights after a HFS metabolic challenge. Consistent with sex differences seen in some epidemiological data, only males in our study had significantly elevated fat mass, whereas females showed a trend toward an increase.

On the other hand, we wanted to test if similar growth-promoting effects are also seen after direct therapeutic antibiotic exposure to young rat pups, therefore we employed a commonly used antibiotic for ear infection (azithromycin) in children with a dosing regimen mimicking human use. Similar to our maternal study, we found increased body weight, fat mass, LPS, fasting insulin levels and insulin resistance with antibiotics, especially in males. Since we employed a different type/dose of antibiotic, phenotypic differences between the two studies were seen. It was shown previously that supraphysiological doses of antibiotics lead to weight loss[142–144], rather than weight gain. Such strong perturbations result in weight loss due to loss of microbiota-derived calories and immune system changes.[21] Therefore, giving a therapeutic dose to animals in our second study directly resulted in greater changes in microbiota composition and greater metabolic impairments, but lesser changes in body composition. Since the duration of the direct antibiotic exposure study was shorter compared to the maternal study, it is possible that a longer HFS metabolic challenge may lead to a stronger phenotype. In addition to the two animal studies, the analysis of human birth cohort data confirmed a significant association between maternal antibiotic exposure during birth and increased infant BMI *z*-score at year 1 of age.

Young rats receiving pulsed antibiotic therapy in our second study had extremely high insulin levels and pronounced insulin resistance, whereas animals in our maternal study had a weaker insulin resistant phenotype as only trends were detected. High LPS levels detected in males in our direct exposure study could explain the differences in insulin resistance as no differences in LPS levels were seen in our maternal study. It has been shown previously that LPS, through binding to TLR-4 increases pro-inflammatory cytokine production (i.e. TNF-α,), which inhibits insulin signalling.[197] Similarly, the altered hepatic and hypothalamic insulin receptor gene expression that we observed in directly-exposed antibiotic males, could also partially explain the insulin resistance seen.

Besides LPS and insulin receptor gene expression, the short-chain fatty acid acetate might drive the insulin resistance seen in our direct-exposure animals, especially because acetate is mostly produced by *Bacteroidaceae*[26] and we observed an extremely high relative abundance of *Bacteroidaceae* in our direct-exposure animals only, whereas no differences in *Bacteroidaceae* were found in our maternal study. Mechanistically, Perry *et al.* (2016) demonstrated that increased acetate production by dysbiotic gut bacteria drives insulin secretion, insulin resistance and obesity via the parasympathetic nervous system (vagus nerve)[279]. In line with this, studies in humans[170,280,281] and mice[218] demonstrated that changes in gut microbiota composition (either with fecal microbiota transplants or antibiotics) impact insulin sensitivity. Similar to our study where we used an antibiotic targeting Gram-positive bacteria, 7-days of oral administration of vancomycin to males with metabolic syndrome, decreased Gram-positive Firmicutes and increased Gram-negatives leading to insulin resistance[170]. The authors conclude that the decrease in insulin sensitivity after vancomycin is due to lack of secondary bile acids and a simultaneous increase in primary bile acids driven by lack of Firmicutes.[170] Firmicutes are known to promote bile acid homeostasis by changing primary bile acids into secondary bile acids, therefore impacting bile acid pools.[282] Apart from the role of bile acids in fat absorption, they also impact insulin secretion/sensitivity through farnesoid X receptor (FXR)/TGR5 with secondary bile acids having a greater affinity for TGR5 than primary bile acids. This finding highlights the link between gut microbiota (Firmicutes), production of secondary bile acids and their role in insulin sensitivity and could partially explain our findings. Future studies should examine fecal concentrations of bile acids as well as fecal and serum concentrations of SCFA to gain a greater understanding of these microbiota-mediated metabolites in contributing to the obese phenotype associated with antibiotic exposure.

We observed similar microbial profiles in males and females after direct antibiotic exposure (lower Firmicutes, mostly *Lactobacillus*) as well as insulin resistance. Another possible mechanism for impaired insulin signalling seen in these animals is the recent discovery that viruses in our gut can produce viral insulin/IGF-1–like peptides (VILPs), which can bind to human insulin receptors and stimulate proliferation and glucose uptake[283]. Further experiments showed that VILPs are present in 30 different species

including humans (feces and blood) and that VILPs produced by viruses can cause or protect from a disease[283]. Even though the affinity of VILPs to insulin receptor is lower when compared to insulin, it is possible that VILPs (produced by dysbiotic microbiota in our direct-exposure animals) competed with insulin to bind to insulin receptors and thus contributed to hyperinsulinemia/insulin resistance. Nevertheless, to understand the precise causes of insulin resistance in our directly exposed males, further studies would be necessary, especially due to the complexity of microbe-host interactions and the possibility of the involvement of multiple pathways.

6.2.2 Antibiotics disrupt maternal microbiota during lactation and impair microbiota establishment in the offspring

Physiological microbiota changes during pregnancy, vertical transmission during birth and establishment and maturation in a newborn/infant are highly complex processes with multiple factors capable of disrupting normal succession including antibiotics.[21] In our first study, high levels of pro-inflammatory *Enterobacteriaceae* and extremely low levels of *Lactobacillus* spp. were found in dams treated with antibiotics. Low *Lactobacillus* spp. levels were reflected in their male offspring, whereas high *Enterobacteriaceae* were seen in female offspring at weaning. The microbial community changes throughout pregnancy, most notably with the third trimester microbiota promoting inflammation, adiposity and hyperglycemia, possibly as an adaptation to the increased energy needs of the fetus.[39] During birth, transmission of vaginal microbiota with high levels of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* spp.[45] should be passed from the mother to the child, however, due to disruption of maternal microbiota with antibiotics, it is likely the offspring in our maternal study were seeded with a dysbiotic microbial composition.

Due to indirect antibiotic exposure via mother and the prolonged HFS metabolic challenge in the offspring, the HFS diet minimized most of the microbiota differences seen at weaning by the end of the study. Nevertheless, it is possible that even subtle microbiota changes were able to promote growth in our offspring. This was shown previously when fecal microbiota from animals treated with LDP could transfer an obese phenotype to germ free mice even before major differences in body fat were seen in the antibiotic-treated donor animals.[130] The authors demonstrated that LDP treated

microbiota induced obesity as well as changed the expression of ileal genes involved in immune system regulation.[130] Immunological changes after LDP such as shortened villi and decreased expression of genes for antimicrobial peptides (i.e. RegIIIγ/Relmβ) and T-helper immune cells (Th17) suggest altered intestinal defences also known to be linked to obesity.[130] Furthermore, a negative correlation between *Lactobacillus* and body weight/fat mass and a positive correlation between *Lactobacillus* and Th17 transcription factor Rorγt and antimicrobial peptides was found.[130] These findings provide a plausible means through which the low *Lactobacillus* levels found chiefly in dams and males in our maternal study reflected stronger phenotypic changes than those seen in females with preserved *Lactobacillus* abundance.

Similarly, in our second study, direct antibiotic exposure led to changes in microbiota development in the offspring over time. Each pulse of azithromycin increased *Bacteroidaceae* in antibiotic-treated groups, whereas levels remained stable over time in control groups. On the other hand, *Lactobacillaceae* decreased over time in control/lean control groups, but steadily increased in antibiotic-treated groups, further demonstrating disruptions in microbiota establishment early in life. Low levels of *Lactobacillus* present early in life in another study led to increased adiposity/weight later in life[130], which is what we observed in both sexes at the end of our direct-exposure study. In addition to obesity risk, early life microbiota disruption with macrolides also disrupts the development of the immune system, however immunomodulation requires pulsed therapeutic level-perturbed microbiota.[228] In a series of experiments, each pulse of macrolide exposure (three in total) led to changes in ileal gene expression, with each subsequent pulse having cumulative effects with changes persisting up to 40 days after a single exposure[228]. After the third pulse of therapeutic dose antibiotic, 148 ileal genes were differentially expressed between pulsed antibiotic and control with reduced intestinal secretory immunoglobulin A (sIgA) secretion and small intestinal lamina propria lymphocytes seen in antibiotic animals.[228] The authors suggest that this immunomodulation with pulsed therapeutic doses of antibiotic could partially explain why children exposed to antibiotics early in life are at higher risk not just for obesity but also for allergic asthma[284], food allergies[285] and type I diabetes.[286] Loss of keystone microbial species early in life due to antibiotic administration allows expansion of

opportunistic bacteria leading to lack of interaction with intestinal epithelium, depletion of homeostatic innate immune system sensors, Th17 cells and ultimately sIgA production.[228] Nevertheless, future studies would be necessary to determine if immunological changes observed in murine models are applicable to humans.

6.2.3 Intrapartum antibiotic prophylaxis increases body mass index z score in the first year of life: results from the All Our Families Pregnancy Cohort

Animal[130,147,204] and several epidemiological human studies[18–20,155,156,165,268] showed a link between early life antibiotic exposure and increased adiposity and weight gain later in life. Nevertheless, the lack of association found in recent studies suggests the link between antibiotic exposure and obesity risk requires further investigation in humans[159–161]. We were fortunate to have access to data from a community-based prospective pregnancy cohort in Calgary, Canada (All Our Families pregnancy cohort [AOF])[260–262] to investigate this link. Our analysis of the AOF cohort indicates that maternal antibiotic exposure during birth (intrapartum), but not during pregnancy, is associated with a significant increase in BMI *z* score at 1 and 2 years of age. After controlling for several known obesity risk factors, the final adjusted linear model showed a significant association between intrapartum antibiotic exposure and increased BMI *z* score at 1 year of age. To our knowledge, this is the first human study to investigate the association between intrapartum antibiotic exposure and infant obesity risk at 1 and 2 years of life.

It is intriguing that we saw an increase in BMI *z* score only at year 1 (but not year 2 and 3) of life with IAP, however, this is not surprising considering this pattern is frequently seen in animal studies[130,204]. Increased early life growth rates (10 days post-weaning) in mice are commonly observed after maternal antibiotic exposure during pregnancy/lactation[130,204]. Even though post-weaning growth rates are then minimized (analogous to our AOF cohort), their obesity risk is revealed when adult animals are challenged with a high fat diet and gain weight at a significantly accelerated rate compared to controls[130]. Therefore, even though the differences in BMI *z* score were diminished at 2 and 3 years of age in AOF cohort, it would be interesting to do a follow up and investigate whether infants exposed to IAP are more susceptible to obesity later in life when exposed to a Western-style diet. Furthermore, an intervention study with a

prebiotic (oligofructose-enriched inulin) supplement in pregnant women or in infants given antibiotics would be highly relevant in determining if the metabolic consequences of antibiotic exposure could be attenuated in humans as shown in rats. The possibility is intriguing given a previous clinical trial showing that the growth rates in children with overweight or obesity are normalized and fat mass reduced after prebiotic intervention.[25]

6.2.4 Oligofructose prevents obesity and improves metabolic profiles when co-administered with antibiotics in dams and offspring

Oligofructose administration in our animal studies resulted in lower body weight and fat mass, improved metabolic profiles (maternal study: increased GLP-1, lower leptin levels; direct-exposure study: reduced insulin and improved insulin sensitivity) and higher levels of health promoting bifidobacteria. Changes in satiety hormones (especially an increase in GLP-1) led to reduced food intake in prebiotic animals, even when antibiotics were co-administered. For example, dams given prebiotics (ABT and ABT+PRE groups) in our maternal study had higher GLP-1 and lower leptin with significantly lower food intake for the duration of the prebiotic intervention. An increase in GLP-1 was probably due to an increased secretion from intestinal L-cells frequently observed after prebiotic administration[287]. Lower food intake would partially explain lower body weight and fat mass in prebiotic animals, however it is unlikely it was the only mechanism. The antibiotic group was significantly heavier than any other group with more fat mass, but there were no differences in food intake or GLP-1 levels between the control and antibiotic group. It is likely that microbiota-dependent mechanisms in antibiotic animals further increased their adiposity and body weight. Furthermore, reduced body weight in our prebiotic animals could also be due to increased SCFA production as a result of prebiotic fermentation. Even though SCFAs are a source of calories, they also play a beneficial role in body weight regulation by targeting several tissues and acting as signal transduction molecules[26]. SCFAs have been shown to modulate the expression of G-coupled receptors and that in turn increase satiety hormone secrtion, reduce body weight, enhance triglyceride hydrolysis, fatty acid oxidation and protect against diet-induced obesity[26].

Maternal prebiotic co-administration with antibiotics prevented the negative outcomes of antibiotics in their offspring. ABT+PRE offspring displayed normalized

early life growth rates, body weight, fat mass, hepatic triglycerides and showed a trend toward an increase in GLP-1 and a decrease in insulin. Even though the offspring were not directly exposed to antibiotics/prebiotics, the impact of antibiotics persisted in the offspring. A possible mechanism for the health benefits seen in prebiotic offspring is altered maternal microbiota transferred at birth as we observed significantly higher levels of health promoting bifidobacteria at weaning in the prebiotic offspring. In addition, it was recently shown that maternal oligofructose supplementation during pregnancy protects the offspring via improvements in serum inflammatory and metabolic molecular profiles as well as fatty acid gene expression and a reduction in hepatic steatosis[213]. Specifically, lower expression of pro-inflammatory genes (i.e. IL-1β, IL-6, MCP) and genes involved in fatty acid metabolism (leptin, PPAR-γ, PGC-1α) was seen with maternal prebiotic intake which could partially contribute to the leaner phenotype of the prebiotic offspring.[213] Changes in tissue gene expression levels were mirrored in serum inflammatory profiles where prebiotic offspring had lower levels of IL-1α, IL-4, IL-1β.[213]

In our direct-exposure animal study, the results were similar; direct prebiotic co-administration with antibiotics in young pups resulted in lower body weight, fat mass and improved insulin sensitivity. It was shown previously that GLP-1 improves glycemic control via increased insulin sensitivity and/or promoting insulin secretion[287], however we did not observe differences in GLP-1 levels between the groups (data not shown) so we decided to investigate additional potential mechanisms. Analysis of hepatic gene expression showed that female prebiotic groups (PRE and ABT+PRE) had increased IRS-1 and IRS-2 expression at the end of the study, however this was not observed in males. Similarly, increased hypothalamic gene expression of NPY/AgRP was seen in female prebiotic groups (PRE and ABT+PRE) only, which would explain why prebiotic females trended towards lower food intake, even when on a HFS diet. While NPY/AgRP is orexigenic and it increases food intake, fasting overnight should lower insulin/glucose/leptin levels and consequently increase the expression of NPY/AgRP to promote feeding[250]. After an overnight fast, we observed low leptin/insulin/glucose and increased expression of NPY/AgRP in our female prebiotic groups only, suggesting that only these groups of animals had hypothalamic circuits intact. Furthermore, since central NPY also controls peripheral systems including pancreatic insulin release, it is also

possible that low fasting insulin levels in prebiotic female groups, could be partially explained by high NPY/AgRP expression. It has been shown that NPY released from sympathetic neurons, which innervate the pancreas, inhibits insulin release[288]. Further experiments would be necessary to confirm these hypotheses.

In both animal studies we observed sex-specific differences and it was shown previously that sex hormones influence disease progression via microbe-dependent mechanisms[289]. Antibiotics had a more detrimental impact on males, whereas prebiotic co-administration had a more positive impact on females. We propose several possible mechanisms. Firstly, metabolic hormones such as leptin, transmit signals to the developing hypothalamus and early life challenges such as HFS/antibiotics might lead to impaired hypothalamic development, which controls energy expenditure and food intake[290]. Since leptin levels at the end of the antibiotic pulses in males in our second study were double when compared to females (absolute values, comparison between the same groups), we hypothesize that high leptin levels could interfere with normal hypothalamic development and therefore worsen their phenotypes. Second, higher LPS levels at the end of the second study were only observed in ABT males, which could lead to production of pro-inflammatory cytokines (i.e. IL-6/TNF- α) and impair insulin signalling[242], therefore explaining why males were more insulin resistant than females. Third, early life prebiotic supplementation in rats has sex specific effects on body composition and metabolic markers as shown previously[291]. Briefly, prebiotics in both sexes reduced fat mass and increased satiety hormone production (GLP-2, pancreatic peptide Y [PYY])[291]. However, only males had reduced body weight, but females displayed greater lean mass and lower LPS levels, suggesting a stronger positive health impact on females[291]. Nevertheless, further research is needed to explain why sex differences occur with antibiotic/prebiotic administration.

6.2.5 Oligofructose when co-administered with antibiotics helps to correct dysbiotic microbiota

Prebiotics promote the growth of *Lactobacillus* and/or *Bifidobacterium* spp.[188], which we observed in both animal studies. In the maternal study at birth, *Lactobacillus* spp. levels increased, whereas pro-inflammatory *Enterobacteriaceae* levels decreased to levels that matched the control group when prebiotics were co-administered with

antibiotics (ABT+PRE) in dams. In addition, dams on the prebiotic diet (PRE, ABT+PRE) had significantly higher levels of *Bifidobacterium* spp. and *Collinsella aerofaciens* and both genera positively correlated with GLP-1 and cecum size (a marker of increased fermentation). Furthermore, *Bifidobacterium choerinum* was negatively correlated with leptin, whereas *Collinsella aerofaciens* negatively correlated with body weight. Our findings are in line with a previous study where increased *Bifidobacterium* spp. and *Collinsella* levels were associated with a lean phenotype[212].

The preceding changes in microbial composition at birth and during lactation were transferred to the offspring with sex-specific differences. Maternal prebiotic supplementation resulted in higher levels of *Lactobacillus* spp. at weaning in male offspring only, however, in females, normalized *Enterobacteriaceae* were seen after maternal prebiotic consumption. At weaning, higher levels of *Bifidobacterium* spp. and *Collinsella aerofaciens* already seen in dams, were mirrored in their offspring in both sexes. Bifidobacteria are extremely important early in life as they are the predominant colonizer of the infant gut, contribute to immune system development and gut maturation and high levels have been associated with reductions in body fat, hepatic steatosis and improved glucose control[196,214,215]. Therefore, it is possible that prebiotic offspring were protected against obesity and metabolic impairments partially due to gut microbiota differences seeded at birth.

Since the second animal study employed a protocol with direct antibiotic/prebiotic exposure, the impact on gut microbiota composition was greater when compared to the first study with indirect exposure. While antibiotics completely depleted *Lactobacillaceae* and changed the course of microbiota maturation, prebiotic co-administration (ABT+PRE) rescued low *Lactobacillaceae* in males and females. Interestingly, improved intestinal permeability, lower LPS levels and decreased body weight/fat mass seen after OFS supplementation were likely due to the presence of *Lactobacillus* and *Bifidobacterium* in the gut given that once their growth was inhibited with antibiotics[206], the health benefits no longer persisted. Therefore increases in *Lactobacillaceae* with prebiotic co-administration in our first and second study could partially explain lower body weight and improved metabolic profiles seen in prebiotic groups when compared to antibiotic groups.

6.3 Strengths and limitations

6.3.1 Animal model

We decided to work with Sprague Dawley rats as this strain has been commonly used in our previous prebiotic and obesity-related studies and the majority of protocols are firmly established[202,206,213]. In addition, the obese phenotype and response to a high fat/sucrose diet of the Sprague-Dawley rat closely resembles the development and comorbidities of human obesity[292]. For example, diet-induced obese (DIO) Sprague Dawley rats mimic hypothalamic NPY gene expression seen in humans with obesity[293]. In addition to altered hypothalamic circuits, other metabolic impairments such as dyslipidemia, hypertension, insulin resistance and hepatic steatosis are seen in DIO Sprague Dawley rats and human obesity[294]. Furthermore, rats have more similar gut microbiota to humans than mice do and since one of our main outcomes was gut microbiota composition, we decided to employ the Sprague Dawley rat model[295]. Given that we required a model where metabolic tests were performed repeatedly (i.e. oral glucose tolerance tests with additional blood collection for satiety hormone measurement and insulin tolerance tests), we had to select animals larger than mice. Based on the volume of blood required for testing, rats were the preferred species given that the total blood volume of rodents corresponds to 6-8% of their body weight and < 10% can be collected per week followed by a 2-week rest prior to the next collection[296].

Even though Wistar and Sprague-Dawley rats are becoming the most commonly used laboratory animals[297], limitations exist. One limitation is that we did not distinguish between obesity prone and obesity resistant rats in our animal studies. When rats are fed a high fat/sucrose diet for 8-10 weeks, obesity resistant and obesity prone phenotypes emerge[202,206]. In study 1, we fed offspring a HFS diet for 8 weeks starting at 9 weeks of age and in study 2, offspring were weaned onto a HFS diet at 3 weeks of age until week 10. Although we saw a strong obese phenotype, particularly in males, it is possible that there were animals that were more resistant to obesity and therefore contributed to greater variability in the data. Another limitation is translating the age of a rat to human years. Several different methods have been employed over the years to correlate the ages of

small mammals to humans (i.e. growth of molar teeth, weight of the eye lens), but none of the methods exactly define the absolute age[297]. Life expectancy of a laboratory rat ranges from 2 – 3.5 years[297], therefore both of our animal studies ended in the first half/third of their life and the question is whether our findings are translatable to human adult obesity. Our maternal animal study lasted 17 weeks and in our direct-exposure study animals were sacrificed at week 10 of life, which is considered young adulthood (adolescence) in rats. Therefore, rats in our studies continued to gain weight throughout our interventions. More accurately, the prebiotics in our studies decreased the rate at which animals gained weight and fat mass rather than actually causing weight loss. Nevertheless, despite employing rats in the adolescent stage of life, our gut microbiota findings, we believe, are translatable to humans especially as we studied vertical maternal transmission (seeding) at birth (study 1) and gut microbiota maturation postnatally (study 2). In humans, the composition of the gut microbiota transitions to an adult-like state by 3 years of life[65], therefore, both of our studies included this time period. Furthermore, evidence from both pediatric and adolescent populations that investigated the impact of antibiotics or prebiotics on anthropometric and metabolic parameters[3,25,194] confirm our findings. Thus, the difference in age between human and rat studies is not likely to significantly influence our outcomes.

6.3.2 Clinical participants

Intrapartum antibiotic use was obtained from obstetrical and birth records which was a major strength of our AOF pregnancy cohort since self-report bias was avoided. On the other hand, major limitations of our AOF pregnancy cohort include: self reported antibiotic use during pregnancy, missing data at year 1 and year 2 of life and categorical (yes/no) reporting (rather than duration) of variables which impact body weight such as smoking/breastfeeding. It has been previously shown that breastfeeding duration is associated with infant's obesity risk with children that are breastfed <6 months having higher growth rates when compared to children breastfed >6 months[298]. Since we found no association between breastfeeding (yes/no) and BMI *z* scores with >95% women answering yes to breastfeeding, it is possible that no association was found because some infants were breastfed for a very short period of time. In addition, we acquired pre-existing data from the cohort study and were therefore not actively involved in

questionnaire development which could have improved reporting on our outcomes of interest. In the future, the collection of the duration of breastfeeding/smoking and the exact type/dose/duration of antibiotic exposure during pregnancy would be necessary to further confirm the data. It is possible we found no association between antibiotic exposure during pregnancy and BMI *z* score if women had a single antibiotic exposure with a narrow spectrum of activity during the first trimester since this type/dose/timing of administration does not appear to increase obesity risk as reported previously where the greatest risk is seen in the second and third trimester and with repeated dosing[257]. Furthermore, collection of cord blood and meconium (the first feces of the newborn) would allow us to investigate cord blood metabolite profiles[299] and gut bacteria seeded during birth[14] as both appear to be associated with obesity and might reflect intrapartum/during pregnancy antibiotic use.

6.3.3 Diet interventions

AIN-93G (growth, pregnancy, lactation) and AIN-93M (adult maintenance) purified diets recommended by the American Institute of Nutrition were used as control diets for our maternal animal study[300], whereas for our second animal study we used normal chow. As the AIN-93 diet is largely composed of readily digestible ingredients (sucrose, dyetrose, cornstarch, soybean oil) and low fibre content[301], it may in fact have an obesogenic effect as has been observed in unpublished work in our lab. As a result, we decided to use normal chow for our direct-exposure study instead, which contains a less processed mixture of crude ingredients (soybean meal, ground corn, porcine meat and bone meal) and only 4% sucrose[197] , whereas AIN93-M has 10% sucrose.

For the prebiotic intervention, we employed a 10% wt/wt dose of oligofructose as this is widely used in animal studies and results in reduced body weight/adiposity and improved metabolic profiles[202,206,302–304]. A limitation of this dose is that 10% wt/wt administration is not normally achievable in humans due to gastrointestinal side effects. Nonetheless, human studies employing lower doses of 8 grams/day for 16 weeks in children[25] and 16-21 grams/day in adults reported similar health benefits as seen in animal studies, although at a much lower magnitude[305]. Since the cecum size in humans is relatively small, it is possible that increased cecum size seen in rats is the evolutionary adaptation to increased fermentation of indigestible food ingredients commonly seen in

their diet[295]. We observed this in our second animal study where cecum size increased after prebiotic administration but was minimized by the end of the study when prebiotic intake has ceased. Another issue arising with 10% wt/wt oligofructose diet is the slight energy dilution that occurs because of the lower energy density of oligofructose (1.6 kcal/g versus 4 kcal/g for digestible carbohydrates). Nevertheless, when we assessed food intake, we accounted for the difference in energy density between the diets and presented calorie (energy) intake as opposed to strictly reporting grams of food intake. In studies where the reduced caloric intake with 10% oligofructose supplementation has been examined in detail, it is has been shown that increased satiety hormone production and a reduction in appetite, rather than energy dilution effect is largely driving the reduction in body weight[202,205]. On the other hand, other fiber sources such as cellulose (non-fermentable fiber used in AIN-93 diets) do not increase satiety hormone production and can result in increased food intake as animals may try to compensate for reduced energy content in the diet which is common in rodents but not necessarily humans[306]. Since we observed increased GLP-1 levels in our maternal study after oligofructose intake, it is likely this satiety hormone contributed to reduced food intake and a leaner phenotype in dams. In our second study GLP-1 levels did not change after oligofructose supplementation, nevertheless, the blood was collected via the tail, which is far less optimal than portal vein blood which drains directly from the gut. Due to the short half-life of GLP-1, its main satiation signals are communicated to the brain through the chemosensitive GLP-1R expressed on the dendritic terminals of the vagus nerve[121]. It is very likely we did not observe differences in systemic circulation in our second study due to the blood collection via tail bleed, whereas in our first study blood was collected via portal vein at sacrifice. GLP-1 levels in our maternal study after oligofructose intake were double when compared to other groups. The reason might be two-fold: the portal vein drains the blood from the immediate source of GLP-1 production (intestinal L-cells) and the blood was collected immediately after the end of the prebiotic intervention as dams were sacrificed at weaning and still consuming the prebiotic. The nature of our direct-exposure study did not allow portal vein collection immediately after the prebiotic/antibiotic intervention (day 39 of life) since we kept the animals alive until 10 weeks of age and portal vein blood collection is terminal.

6.3.4 Antibiotic intervention

Our maternal study used sub-therapeutic antibiotic treatment (STAT) as previously reported in other animal studies[130,141]. The reason we decided to use STAT in our first model is the robust obese phenotype with metabolic and immune impairments[130], which results after such administration. In order to investigate the potential for prebiotic to mitigate obesity risk associated with antibiotics, we needed a predictable model to test our hypothesis in. Furthermore, when deciding on the type of antibiotic to use, penicillin appeared to produce the strongest obese phenotype in existing research whereas vancomycin, penicillin plus vancomycin or chlortetracycline did not exhibit as strong of an impact on body weight[141]. In addition, rats resemble humans more closely than mice in pharmacokinetic parameters such as penicillin bioavailability and elimination half-life thereby making penicillin investigation in rats the preferred model[307].

Low doses of antibiotics have been used in farm animals for many decades[308]; therefore it is not surprising we also saw a strong obese phenotype in our maternal study. Despite the value of sub-therapeutic doses in understanding the mechanisms by which early life antibiotic administration affect metabolic health and providing proof of concept that prebiotics can interrupt the establishment of antibiotic-induced obesity, they are limited in that such low doses are normally not administered to women during pregnancy/lactation or to infants and children. For that reason we decided to employ a direct therapeutic dose of macrolide (azithromycin) to young rats in our second animal study. Furthermore, we mimicked a pediatric protocol (once-a-day dosing 10mg/kg/day, 3 day pulses, 3 courses) which is commonly used in human children as well as rodents[136]. Although one study used another macrolide (tylosin tartrate), this type of antibiotic is exclusively used in veterinary practice and only with extremely high doses (50mg/kg/day)[147]. While such a high dose of tylosin resulted in several immune impairments, our main outcome was obesity/gut microbiota/metabolic changes, therefore we decided to use an antibiotic (azithromycin) which is used in humans and rodents with a dose that was as low as possible but still therapeutic. Nevertheless, other limitations with therapeutic dosing still exist in particular that our animals were not actually sick when the antibiotics were given.

6.3.5 Gut microbiota and brain tissue analysis

Gut microbial profiling was completed via qPCR in our first study (chapter 3) and with 16S rRNA amplicon Illumina sequencing in both animal studies (chapter 3 and 4). 16S rRNA amplicon Illumina sequencing was performed on the MiSeq Illumina platform at the Centre for Health Genomics and Informatics (University of Calgary). Although qPCR is quantitative and can be performed easily in the lab without the need for expensive sequencing equipment, Illumina sequencing allows for investigation of global changes in the gut microbial community[309].

As the 16S rRNA gene is highly conserved across bacterial species and it contains variable phylogenetic regions, sequencing of select variable regions (typically V3 and V4) allows for the identification of bacterial species within a sample[309]. Advantages of 16S rRNA Illumina sequencing include: the possibility of identifying lesser known bacteria, information about community characteristics (alpha-diversity, beta-diversity) and information about relative abundances of certain bacterial groups (species). In short, when compared to qPCR which targets specific pre-determined bacterial groups, more information can be gleaned from sequencing based technologies. In our maternal study, sequencing was performed on cecal matter as it is the site where fermentation is most active and the collection point is extremely easy to identify at necropsy. Nevertheless, a limitation of such collection is that only one time point is available for collection as cecal matter collection is a terminal procedure. Therefore, for our second animal study, we collected fecal matter for 16S rRNA Illumina sequencing instead as it allowed us to investigate longitudinal changes in gut microbiota after each antibiotic/prebiotic pulse. A limitation of the fecal matter collection was that it was collected overnight and the samples were at room temperature for some time. Upon collection it was frozen at -80°C within 1-2 hours after collection, whereas cecal matter was snap frozen in liquid nitrogen immediately. Nevertheless, a study showed that leaving the fecal samples for 3 days at the room temperature does not substantially influence the gut microbiota assessment when compared to immediate freezing at -80°C[310], therefore it is very unlikely our samples were compromised with an overnight collection. qPCR analysis (fecal matter) in the first study allowed us to look at quantitative changes in key bacterial species over

time which allowed us to identify some distinct differences during the prebiotic/antibiotic intervention. This finding convinced us to use longitudinal fecal sampling for our second study and employ a more in-depth method of analysis (16S rRNA Illumina sequencing). The strengths of our 16S rRNA Illumina sequencing analysis were that sequences were checked for quality, trimmed, merged, and checked for chimeras using the DADA2[232] and phyloseq[207] packages for R (R Development Core Team; http://www.R-project.org). In addition, to reduce biases introduced by DNA amplification (i.e. PCR) and sequencing errors, we excluded any Amplicon Sequence Variants (ASV) that were found less than five times in the community matrix.

The use of real-time PCR (RT-PCR) for tissue gene expression analysis allows for the quantification of the amount of cDNA (reverse transcribed from RNA), relative to housekeeping gene. For hepatic gene expression analysis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as GAPDH is one of the most commonly used housekeeping genes in the liver and it expression is not influenced by age or gender[311]. For the hypothalamus, beta-actin was selected as a previous study showed a greater stability when compared to GAPDH[312]. Although we detected hypothalamic and hepatic changes in insulin receptor substrates expression with RT-PCR, further studies and different methods would be necessary to demonstrate the true mechanistic involvement of this pathway. Since hypothalamic phosphatidylinositol 3(PI3)-kinase/Akt pathway mediates the effect of insulin and energy expenditure, hypothalamic Akt phosphorylation should be measured in the future using Western blots.

6.3.6 Glucose measurement

Intraperitoneal insulin (ITT) and oral glucose tolerance tests (OGTT) were employed to investigate insulin sensitivity and glucose responses. In our maternal animal study (chapter 3), both tests were employed and since we found no differences in OGTT, we decided to only perform ITT in the second direct-exposure study (chapter 4). While the gold-standard method to assess insulin sensitivity still remains the eugycemic-hyperinsulinemic clamp[229], it was impossible to perform it in our studies due to the large number of animals. Instead, OGTT was chosen over intravenous glucose tolerance test (IVGTT) as it provides a more physiological route of glucose administration and a good assessment of insulin secretory patterns[229]. To minimize stress and avoid injuries, light

anaesthesia was used before gavaging animals with glucose, however that is a limitation of our OGTT testing. It was previously shown that OGTT tests on anasthetized mice yield supraphysiological glucose responses as heart rate and blood flow are affected[313]. Nevertheless, our animals were not completely unconscious and only lightly anesthetized to minimize the stress of the gavage, therefore reducing the likelihood of our results being compromised. An advantage of our ITTs was a 5-hour morning fast, which is less stressful for rats when compared to an overnight 14-16 hour fast normally used in rodent studies[313]. In previous studies in our laboratory where we employed an overnight fasting protocol, some of our rats would become hypoglycaemic during the ITT (especially prebiotic groups) and the test had to be stopped and glucose injected. Another strength of our metabolic testing was that all of the tests were performed during the same time of the day as changes in circadian rhythms affect glucose metabolism[314].

6.4 Future directions and perspective

Antibiotics revolutionized the field of medicine and saved millions of lives. Despite being effective in treating bacterial infections, their use comes with a price. One of the negative side effects of exposure to antibiotics is obesity, which is a world wide epidemic with rates on the rise in Canada.[1] Childhood obesity and overweight represent a serious medical challenge as paediatric obesity increases the risk of obesity in adulthood[315]. Many serious diseases such as metabolic syndrome and cardiovascular disease start to manifest in childhood and worsen with time[315]. While many factors including gut microbiota play a role in the pathophysiology of obesity, microbes also represents an exciting target for novel interventions to prevent obesity.[35] While antibiotics disrupt microbial composition and predispose to obesity, prebiotics reduce body weight via an increase in health promoting bacteria (i.e. bifidobacteria) and improve satiety hormone secretion, to mention a few.[35]

The research findings from our AOF pregnancy cohort study highlight the need for a non-invasive intervention to reduce antibiotic associated obesity risk. We demonstrate that maternal IAP is associated with increased BMI *z* scores at 1 year of age and future studies should investigate the potential to reduce this metabolic risk. Since prebiotics are safe to use with minimal side effects, randomized control trials investigating the potential of prebiotic co-administration with antibiotics are warranted to

determine if this easy-to-administer intervention could be employed to reduce the burden of childhood obesity. In addition, meconium and cord blood should be collected after birth as they represent non-invasive biological samples that could inform future research. Information acquired from meconium and cord blood would have the potential to identify the underlying mechanisms of how IAP predisposes to obesity. For example, a recent longitudinal study compared cord blood metabolite profiles of infants with rapid weight gain early in life (0-6 months) and mid-childhood obesity (median 7.7 years) with normal postnatal growth controls[299]. The results showed that 16 out of 415 detected metabolites are associated with rapid postnatal weight gain. Since it is known that IAP attains sufficient antibiotic levels in the fetal bloodstream during labour[254] and it is unknown how antibiotics present in the cord blood change metabolite profiles, measurement of these metabolic signaling molecules would be undoubtedly informative. Does cord blood of infants exposed to antibiotics during birth mimic the metabolite profiles that are linked to rapid postnatal weight gain? Are there any unique metabolites that are present in antibiotic treated infants only? Cord blood metabolomics analysis and measurement of metabolic markers (i.e. leptin and adiponectin) could answer these and other questions. Similarly, collecting meconium from the newborn would give us information about the vertical transmission of bacteria during birth and answer whether the starting point of gut bacteria seeding at birth differs from non-IAP controls. To gather information about gut microbiota maturation, longitudinal fecal samples should be collected and analyzed. Having a group with maternal IAP and prebiotic co-administration would answer whether we could prevent/reduce the negative outcomes of IAP administration also in a human cohort.

Both of our animal studies demonstrate the potential of prebiotics to reduce body weight, improve metabolic and gut microbiota profiles. Nevertheless, the mechanisms by which this occurs remain elusive and should be investigated. One methodological advancement to add to future studies is fecal microbiota transplant (FMT) early in life. We performed FMT in our first study and showed that microbiota from 17 week old rats whose mothers consumed prebiotic during pregnancy and lactation promoted a lean phenotype characterized by reduced growth rates over the 22 day experiment. However, due to the indirect antibiotic/prebiotic exposure, the phenotypes were not as robust as

might been expected, especially as the antibiotic exposure was indirect and a subterapeuthic dose was utilized. In the future, microbiota from earlier time points (e.g. immediately after weaning) should be transferred to add to the information gleaned from our FMT with week 17 cecal samples. It is likely that the main limitation of our FMT study was the selection of the samples for transplant given that by week 17 of life the HFS diet had blunted most of the differences in microbial composition observed at weaning. However, since we did not collect cecal matter at weaning, this experiment simply was not possible, but should be done in future studies. If gut bacteria are causative in contributing to obesity seen after antibiotic exposure, FMTs after direct antibiotic exposure should be performed as they are currently the gold standard means to show a mechanistic link between obesity and early life antibiotic exposure. Low *Lactobacillus* abundance in both animal studies after antibiotic exposure as well as high pro-inflammatory *Enterobacteriaceae* levels (maternal study) early in life suggest an involvement of gut microbiota, however, we cannot exclude the possibility of other pathways. Furthermore, despite microbial changes seen after antibiotic exposure in both of our studies the question remains whether functional microbiota changes were also present. Future studies should perform shotgun sequencing where in addition to the microbiota composition, the metabolic potential of the microbes can be investigated, especially as functional redundancy is present among microbes[316]. Furthermore, to fully assess metabolic status, high-throughput methods such as mass spectrometry and nuclear magnetic resonance spectroscopy analysis in feces, urine and blood would allow identification of altered metabolites. Even if the microbial composition remains stable after xenobiotic exposure[317], the physiology (metabolic profile) of the individual might be changed, therefore measuring just microbial abundance is not sufficient[316].

Moving forward, our daily prebiotic intake should increase regardless of our past antibiotic exposure. It is well-accepted that a Western-style diet rich in saturated fats and sugar can lead to possibly irreversible health consequences with each generation increasingly struggling to repair the damage. As the gut microbiota is a living ecosystem, environmental insults (i.e. diet, antibiotics) pose a threat which was clearly demonstrated in our animal studies. Experimentally it has been shown that switching from a low-fat, plant polysaccharide-rich diet to a low-fibre, high-fat, high-sugar Western diet, triggered

massive shifts in microbial composition/function that were detectable after one day, even before any phenotype emerged[318]. What is even more concerning is that a subset of bacteria in our gut has the ability to remember past diets[173] meaning their function does not only depend on a current diet, but also on past diets. Sonnenburg et al. (2016) demonstrated that low fibre intake results in a loss of keystone microbial species and this deficiency is transferred and magnified over generations[176]. When a 4th generation of mice was re-introduced to a high-fibre diet, the lost gut microbial species were not regained and only FMT was able to improve the gut microbiota profiles back to those that matched the control group[176]. Furthermore, loss of these keystone species also resulted in the loss of fibre-degrading capacity (i.e. loss of glycoside hydrolase repertoire)[176]. Taken together, evidence from animal studies such as these and others suggest that it is critical that we increase our fibre intake as well as avoid a Western style diet in order to prevent the loss of keystone species that are important for our health over time. Furthermore, there is growing concern with the use of low calorie sweeteners (LCS) to reduce daily caloric intake based on large epidemiological studies demonstrating unintended consequences of LCS consumption such as the development of metabolic syndrome, cardiovascular disease, higher infant BMI and adult obesity[319–324]. Animal studies suggest changes in gut microbiota and gut-brain axis as the key players in the adverse health outcomes after LCS consumption[325,326].

A favorable step forward toward the promotion of foods that favor health and potentially also the gut microbiota was recently enacted in Canada, where a new food guide, based on science and not industry influence, was published[327]. The new guide recommends water as the drink of choice (no LCS), plenty of vegetables, fruits and whole grain foods (excellent source of fibre) and mostly plant protein based diet[327]. This is extremely important for two reasons: first, animal research with early life antibiotic exposure shows that unless animals are challenged with a Western-type diet later in life, they do not become obese[130,141] and second, if we as a society don't consume sufficient dietary fiber, we are at risk of losing keystone microbial species over time as suggested in the eloquent animal study by Sonnenburg[176]. Loss of the keystone species will probably require either probiotics or FMTs to restore the lost species, however FMTs do not come without risks. Recently, The Food and Drug Administration (FDA) released an important

safety alert and stopped all clinical trials in the USA involving FMTs after the death of a patient[328]. Two immunocompromised patients received FMTs from the same donor, but the fecal matter was contaminated with multi-drug resistant microorganisms[328]. Specifically, extended-spectrum beta-lactamase-producing *Escherichia coli* (*E.coli*) resulted in a death of one the patients as they became resistant to a broad range of antibiotics such as beta lactams, including third generation cephalosporins[329]. Therefore to avoid the risks of such extreme measures as FMTs, obesity prevention strategies including lifestyle changes with a high prebiotic diet should be implemented in our everyday lives prior to disease manifestation. Furthermore, our baseline microbiota composition/function has the potential to determine our response/non-response to interventions. Last but not least, additional studies investigating microbiota modulators such as LCS and antibiotics are warranted in order that potential health risks can be appropriately declared on food packaging and drug inserts.

6.5 Conclusion and significance

In summary, the basic and clinical research presented in this thesis support the evidence that antibiotics increase obesity risk and impair metabolism. In our human study, an increased infant BMI *z* score at year 1 of life was associated with maternal IAP intake (chapter 5). Similarly, in our first animal study (chapter 3) maternal antibiotic intake resulted in increased risk of obesity in dams and their offspring. Our second animal study confirmed these findings and showed that direct antibiotic exposure to young rats resulted in increased body weight and metabolic impairments (insulin resistance). Furthermore, animal studies provide evidence for the benefit of the prebiotic oligofructose to reverse the obesity risk and metabolic impairments caused by early life antibiotic exposure. Since obesity is a multifactorial disease, it is not surprising that the mechanisms involved are diverse. Nonetheless, we show that with prebiotic co-administration with antibiotics we improve satiety hormone profiles, reduce liver triglycerides, body fat, hepatic/hypothalamic gene expression and body mass possibly through microbiota-dependent mechanisms. These findings should be investigated in future clinical trials, where ultimately, prebiotic co-administration with antibiotics early in life could be one of the strategies to reduce the burden of obesity.

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