Perinatal Antibiotic Prophylaxis—Friend or Foe?

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Project Summary/Abstract

The human microbiota is the community of organisms that live in and on the human body and interacts with the host to influence metabolism and immunity. These organisms evolve within the host throughout the lifespan impacting growth and immune regulation. The microbiota is established first from the mother during gestation and develops over the first few years into the adult human pattern. Infants whose colonization is disrupted by antibiotic exposure have altered microbial composition that may persist for years (Mueller, Bakacs, Combellick, Grigoryan, & Dominguez-Bello, 2015). These years represent critical developmental periods that may change growth patterns and immune function across the lifespan. Prophylactic antibiotics are administered to the 1.2 million women receiving cesarean sections, and to the 975,000 pregnant women who are colonized with Group beta streptococcus in the United States each year (Hamilton, Martin, Osterman, Curtin, & Matthews, 2015). Febrile women and those with prolonged rupture of membranes, or other risk factors are also treated prior to delivery. While studies indicate that antibiotics administered to the infant have lasting effects on microbial colonization and are associated with obesity and asthma, the impact of prophylactic antibiotics administered to the mother on the infant microbiome has not been well studied. In this study, we seek to understand the impact of antibiotics administered to pregnant women on the microbiota of their infants and the resultant effect on growth and resistance to infection. We will use a murine model to minimize confounders such as genetic, diet, and environmental variation, and to allow tissue specific examination of microbial colonies and the effects that altered colonization may

elicit to the host. It is important to understand the changes that occur in the developing newborn because of antimicrobial prophylaxis to the mother, as these changes occur at a critical period, when the newborn is first being colonized. As microbial colonization influences growth patterns, immune function, and behavior of the host, and the incidence of childhood asthma, obesity, allergy, and autoimmunity are increasing, learning the relationship between these pathologies and common elective therapies, such as antimicrobial prophylaxis is essential.

Project Narrative (short)

Dysbiosis has been linked to obesity, allergy, and autoimmunity. Antibiotics administered during pregnancy may alter microbial thereby negatively altering infant growth and immune function. The proposed study will use a murine model to elucidate the short and long-term effects of antimicrobial administration during pregnancy on offspring microbiota, thus providing a framework to study these effects in pregnant women and their infants.

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Specific Aims/Impact

Twenty-five percent of all women are colonized with *Group Beta streptococcus* (GBS). Current CDC recommendations are that pregnant women colonized with GBS should be treated with antibiotics at least 4 hours prior to delivery to prevent early onset GBS sepsis in the neonate (Verani, McGee, Schrag, & Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention (CDC), 2010). Other indications for prophylactic antimicrobial administration during pregnancy include prolonged rupture of membranes, maternal fever, and premature labor. Although this practice has reduced the incidence of early onset GBS sepsis, gram negative infections, including the most common cause of neonatal mortality, *Escherichia coli* sepsis, have increased (American College of Obstetricians and Gynecologists, 2011). Furthermore, the long-term effects of this practice on neonatal development have not been well studied.

The advent of genomic analysis has led to the recognition that environmental factors influence gene expression and therefore impact human development, function, and behavior (Diaz Heijtz, 2016; Gensollen, Iyer, Kasper, & Blumberg, 2016; Wang, Monaco, & Donovan, 2016). One such environmental factor is the microbial milieu that colonizes the infant during early life. The postnatal colonization process effects the developmental programming of epithelial barrier function, gut and immune homeostasis, and angiogenesis

(Diaz Heijtz, 2016). Antimicrobials administered to the mother during pregnancy, therefore, may initiate long-term effects on infant immunity, metabolism, and behavior. To date research has largely focused on the role of microbiota perturbations as a result of

ingested antibiotic administration. While studying the direct effects of antimicrobial administration is important, establishing the impact of antibiotic prophylaxis during gestation will elucidate the effects of maternal dysbiosis on initial infant colonization, and the resultant impact on developmental programing. The *long-term goal* is to determine the role of initial microbial colonization on metabolic and immune function. The *objective of the present study* is to determine how antibiotics administered to the mother during parturition impact the establishment of a commensal microbiota in offspring. The *central hypothesis* for the proposed project is that antimicrobials administered during pregnancy prevent the full spectrum of commensal colonization, resulting in altered metabolic and immune programming. The *rationale* for the proposed research is that understanding the impact of prophylactic antibiotics on the microbiota during development may lead to more targeted prophylactic approaches, and the ability to modulate microbial composition to promote advantageous immune and metabolic programming.

Aim 1: Determine the impact of intrapartum antimicrobial administration during parturition on the microbial community during the development of offspring. The *working hypothesis* of this aim is that induced maternal dysbiosis alters offspring commensal colonization throughout the developmental period. Metagenomic analysis (16S rRNA, metagenome and microbial sequencing) will be performed on C57BL/6 mice during pregnancy and throughout offspring development in alimentary compartments.

Aim 2: Investigate the functional consequences of dysbiosis during immunological development on offspring growth and resistance to infection. The

working hypothesis of this aim is that altered commensal colonization during development impacts metabolic and immune programming, resulting in malnutrition, obesity, and susceptibility to infection. Fecal transplantation of a *Bacteroidales* and *E. coli* consortium will be performed to determine the functional impact of a dysbiotic microbiota. *Citrobacter Rodentium* infection and peritonitis will be induced to assess immune function.

At the completion of this study, microbial communities associated with obesity, stunted growth, and greater susceptibility to infection will be identified. These findings will be important because they will identify novel pathways that can be targeted to prevent early malnutrition and infection in response to dysbiosis during the developmental period.

Research Strategy

Significance/Background

The nearly sterile fetus is born into a massive microbial community and has evolved to coexist with these microbes at all environmental interfaces. Over the first few years, the infant gradually establishes a microbiota resembling the adult. Microbes in the intestinal tract interact symbiotically with the host to influence structural, metabolic, and immunologic development (Cunningham, Kramer, & Narayan, 2014; Kamada & Núñez, 2014). Alterations to the neonatal intestinal microbiota lead to changes in morphology including villus architecture, blood vessel density, mucus layer properties, and the growth and proliferation of lymphoid tissues (Sommer & Bäckhed, 2013). Microbial dysbiosis and lack of diversity can lead to childhood obesity or malnutrition secondary to alterations in metabolism resulting from reduced metabolic capacity and adaptability (Langdon, Crook, & Dantas, 2016; Tilg & Adolph, 2015). Obesity has reached epidemic proportions in developed countries where antibiotic administration, and the subsequent intestinal microbial alterations, are common. Microbes from obesity-discordant twins can induce obesity discordance in gnotobiotic mice (Ridaura et al., 2013), suggesting that the microbiota can influence metabolic pathology. Conversely, antibiotic administration in addition to nutritional therapy can improve recovery and reduce mortality associated with severe malnutrition (Trehan et al., 2013). These findings suggest not only a microbial role in the establishment of normal metabolic development, but also a role in altering pathologic phenotypes.

In addition to metabolic programing, the microbiota impacts immune health by influencing expression of pattern-recognition receptors on epithelial cells and by

secreting their ligands, which induce the expression of cytokines that shape the composition of the innate and adaptive immune system (Elson & Alexander, 2015). Resident microbes, epithelial cells, and local immune cells interact to foster local and systemic immune homeostasis (Sánchez-Cañizares, Jorrín, Poole, & Tkacz, 2017). Interactions between the gut microbiota and the developing structural and functional immune system result in altered resistance to infection and both protection from and propensity toward autoimmunity (Yurkovetskiy, Pickard, & Chervonsky, 2015). Alterations to the microbiota during initial colonization and the critical period when the adult microbiota is established may be especially detrimental as the opportunity to influence immunologic programming is at its peak.

As changes in microbial colonization impact neonatal developmental programing leading to profound effects on the structure and function of many systems in later life, the common practice of prophylactic antimicrobial administration to pregnant women and neonates deserves scrutiny to determine if the short-term gains in reduced postpartum and early onset neonatal sepsis outweigh the long-term losses in immunity and metabolism.

Innovation

This proposal is highly innovative both conceptually and experimentally. First, this project will provide great insight on the contribution of the early microbiota on obesity and resistance to infection. Research in this area has largely been limited to the direct effects of antibiotic administration on juveniles throughout development using orally administered antibiotics in the drinking water of mice. In this study, antibiotics will be administered both orally and intraperitoneally, exclusively to pregnant mice, which will elucidate the effects of maternal dysbiosis during the colonization period of young

mice while not exposing them directly to antibiotics. This will more closely simulate the experience of infants whose mothers may receive antibiotic prophylaxis administered during parturition. Second, this project will provide insight on the long-term metabolic and immune effects of colonization with reduced diversity as seen in developed nations.

From an experimental perspective, antibiotic treated pregnant dams residing in a specific pathogen-free environment will be used to generate pups whose microbiota will be characterized using 16S rRNA genes captured from the stomach, ileum, colon, and stool at weekly intervals from birth to sexual maturity giving a unique perspective on the progression of microbial colonization in murine offspring. Metagenomic sequencing will provide insight, in terms of the genomic landscape of the microbial ecology in each compartment. An enteropathy-inducing bacterial consortium will be introduced to understand the effects of dysbiosis during the murine developmental period. These pups will then be challenged metabolically with a Western Diet and immunologically with infectious organisms or bacterial endotoxin to reveal the long-term outcomes of early dysbiotic colonization on growth and immunity. This innovative project is supported by a team with expertise in microbiology, immunology, metagenomic and transcriptional sequencing, and bioinformatics.

Approach

C57Bl/6 pregnant mice will be treated with oral antibiotics for 7 days or intraperitoneal antibiotics 1-2 days prior to delivery. Pups will be weighed weekly. Mothers' stools will be collected before and after antibiotic administration to document the change in the microbiota in response to antibiotic administration during pregnancy. Mother and pup stools will be collected before and after weaning, and offspring stomach,

intestine, and colon will be collected before and after weaning to document the microbiome present in offspring during development. Offspring will be placed on a western diet containing 35% fat (10% saturated), 50% carbohydrates (15% fructose), 15% protein, and 16g fiber. Gastrocnemius muscle and mesenteric adipose compartments will be compared at 10, 20, and 30 weeks. Ileum, colon, liver, and mesenteric lymph nodes will be weighed and collected at 10, 20, and 30 weeks to determine the metabolic and immune functional differences that correlate with differential microbiota. Offspring will be injected with lipopolysaccharide, cecal slurry, or gavaged with *citrobacter rodentium* to determine the effects of dysbiosis during development on immune response to sepsis, peritonitis, and transient gastrointestinal infection. Blood, mesenteric lymph node, and spleen will be examined post infection to determine altered immune responses in the setting of dysbiosis during development.

Overview

	Gestation			Nursing	Weaned to sexed cages. Start Western Diet
Control	conception			Weigh weekly, collect stool from mother and pups before antibiotics and before and after weaning.	Weigh weekly. Collect stool and harvest liver, heart, colon, ileum, gastrocnemius, and mesenteric fat @ 10, 20, and 30 weeks. Glucose tolerance test and Insulin levels at 9, 19, 24, and 29 weeks
MAG-C (in drinking water)			Vancomycin, Doripenem, Neomycin		
MAG-H (intraperitoneal)			Ampicillin, Cefazolin		
Timeline	-3 weeks	-2 weeks	-1 week [-1day]	0-3 weeks	3 weeks-30 weeks

	Gestation			Nursing	Weaned to sexed cages at 4 weeks.
Control	conception			Weigh weekly, collect stool from mother and pups before antibiotics and before and after weaning.	Weigh weekly. Administer 7mg/kg LPS or ceal slurry 0.2ml IP x 1 or c. rodentium OG x 5days. Harvest blood, mesenteric lymph node, spleen
MAG-C (in drinking water)			Vancomycin, Doripenem, Neomycin		
MAG-H (intraperitoneal)			Ampicillin, Cefazolin		
Timeline	-3 weeks	-2 weeks	-1 week [-1day]	0-4 weeks	6 weeks

PRELIMINARY DATA:

A pilot study was completed wherein 5 pregnant female C57BL/6 mice were given vancomycin and doripenem in drinking water for 7 days prior to delivery. Feces were collected from treated dams and controls before and after antibiotic administration, and from dams and pups before and after weaning. Treated dams demonstrated reduced

alpha diversity and increased beta diversity when compared to controls throughout offspring development (Fig. 1 and 2).

Figure 1

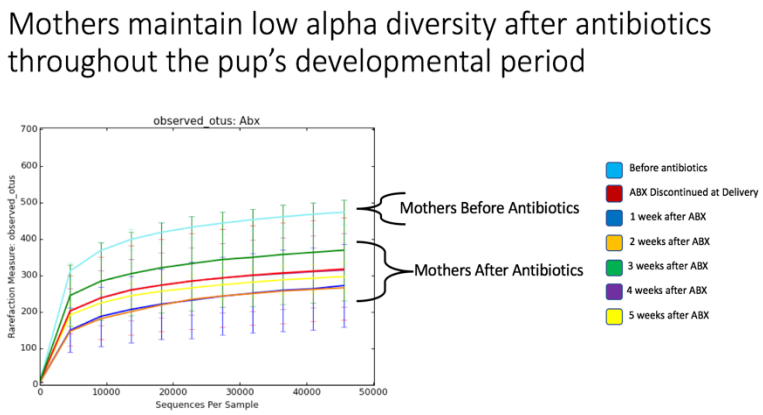
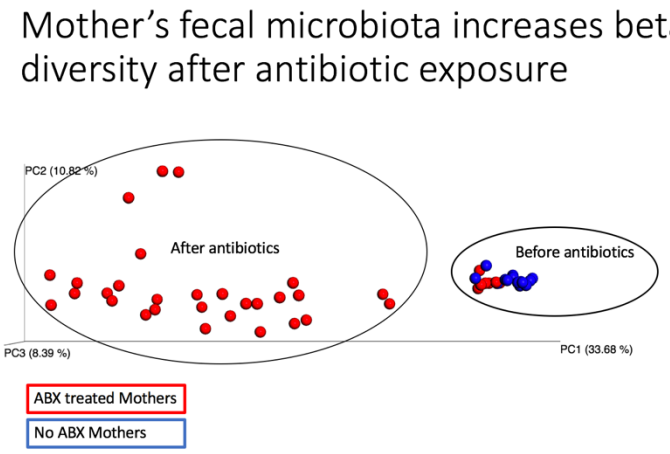
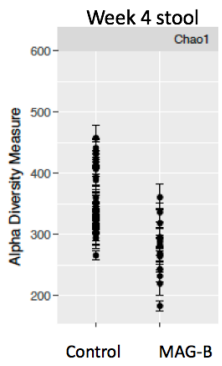


Figure 2

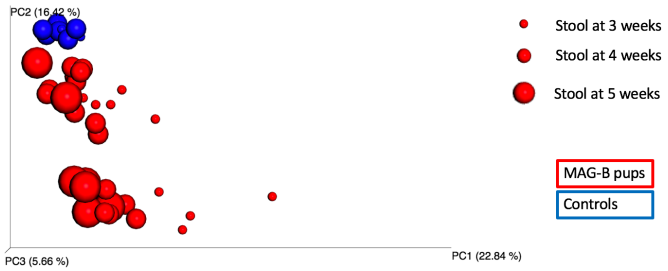


Microbiome research indicates that the composition of the microbiota is altered by external forces such as antibiotic exposure, diet, and inflammation (Lewis et al., 2015) (Bokulich et al., 2016). The impact of maternal dysbiosis on offspring, however, has not been adequately investigated. Our pilot study indicates that offspring reared during maternal dysbiosis after antibiotic treatment had reduced alpha diversity and increased beta diversity compared to the offspring of untreated females (Fig. 3).

Figure 3



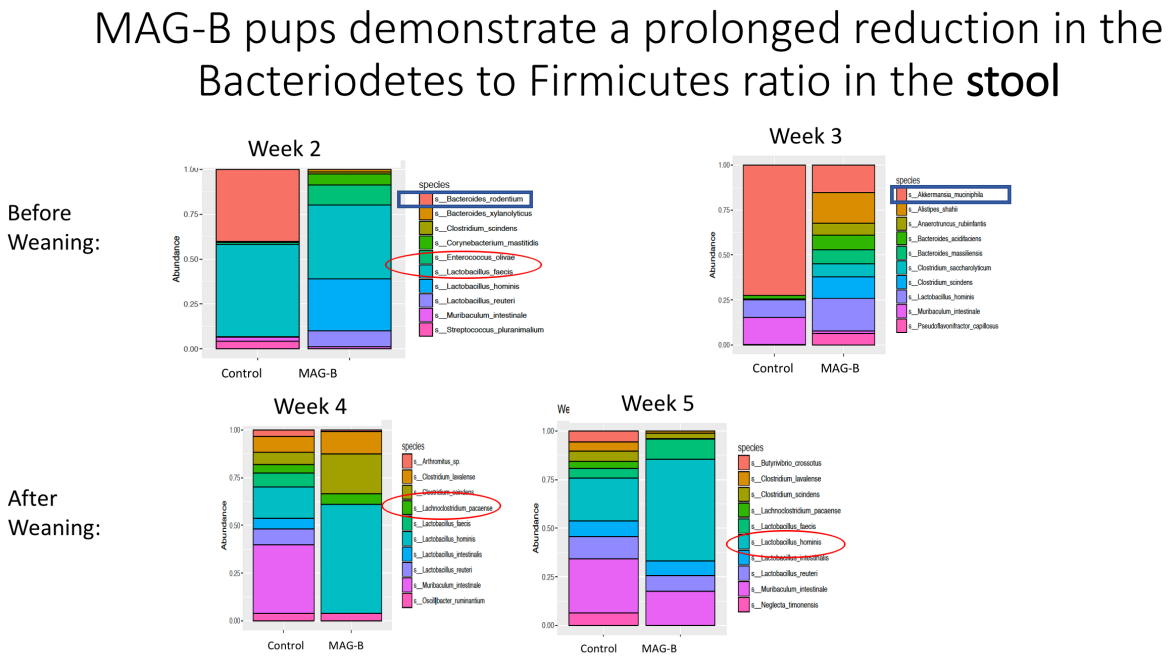
MAG-B pups colonize with different species, and these differences last through maturity



Because antibiotic administration during development is associated with insulin resistance and obesity (Cox & Blaser, 2015), we will investigate whether antibiotics administered to the mother during the last trimester of gestation modifies insulin sensitivity and obesity in her offspring by exposing the offspring to a western diet. The composition of the microbiota that leads to the adverse outcomes associated with obesity are not well understood. Our pilot study indicates that antibiotics administered to the mother during gestation result in decreased Firmicutes and increased Bacteriodetes populations in offspring, and that this altered community composition continues through sexual maturity. A predominance of certain species, such as *B. rodentium* and *A.*

muciniphilia is missing, and the introduction of alternate species such as *enterococcus* occurs during the establishment of solid food ingestion, in the offspring of antibiotic treated mice leading to significant taxonomic differences after weaning (Fig 4). Normally colonized mice develop a stable biome after weaning, while the dybiotic biome fluctuates. These differences may establish lifelong alterations in both metabolism and immune programing.

Figure 4



RESEARCH DESIGN AND METHODS:

Aim 1: Determine the impact of intrapartum antimicrobial administration during parturition on the microbial community during the development of offspring.

1.1 Rationale and general design

Antibiotics produce lasting changes to the gut microbiota when administered during the developmental period that impact host metabolism and resistance to infectious disease (Lange, Buerger, Stallmach, & Bruns, 2016). Although the direct effect of orally administered antimicrobials on the gastrointestinal tract microbiota has been demonstrated (Zhang, Huang, Zhou, Buckley, & Wang, 2013), the effects of administration during pregnancy have not been well studied. Delivery mode has been shown to influence microbiota composition of newborns (Fooladi et al., 2013), but the independent effects of intrapartum antibiotic administration are difficult to discern in human studies. Our preliminary data show that the microbial community composition changes resulting from antibiotics administered to pregnant dams, and alters the colonization patterns of offspring throughout the developmental period, both before and after the establishment of independent feeding (Fig 4). From these findings, it is reasonable to hypothesize that antibiotics administered to the mother during pregnancy induces a dysbiosis, which influences the colonization of her offspring, and that this initial colonization impacts the microbial community composition that is established throughout the developmental period. The objective of this aim is to determine the impact of oral and systemic antibiotics during the last trimester of pregnancy on the commensal colonization of offspring. We will collect feces and vaginal mucosal samples from pregnant female C57Bl/6 mice before and after antibiotic administration during the last days of pregnancy, and then collect weekly fecal samples from both dams and offspring until the offspring are weaned. Offspring stomach, ileum, colon biopsies, and fecal samples will be collected weekly until sexual maturity at age 6 weeks. We will sequence the microbial 16s ribosomal gene using the MO Bio system to document the vaginal and

fecal microbial community structure of the mother, and the gastrointestinal and fecal microbial communities of the offspring. We will perform metagenomic sequencing of DNA from mice with specific bacterial clusters as classified by 16s rRNA sequencing using the Illumina Genome Analyzer system of whole-genome shotgun sequencing. The overall goal of these studies is to identify bacteria and bacterial-derived genes that are differentially present in offspring born to dysbiotic mothers, to determine the gastrointestinal compartments that are distinctly colonized, and to detect the duration of altered colonization. This new knowledge could be utilized to target microbial species and gastrointestinal compartments for therapeutic intervention in the setting of dysbiosis during development.

1.2 Experimental Design:

1.2.1 Measure the impact of antibiotics during gestation on maternal microbiota at delivery.

In preliminary data, we showed that antibiotics administered during late gestation perturbed the maternal microbial environment, and that these changes lasted for the weeks that encompassed offspring microbial acquisition and colonization (Fig 2). Furthermore, we demonstrated that the microbial environment of antibiotic-exposed mothers had reduced alpha diversity when compared to un-exposed mothers (Fig 1). In these experiments, we will administer oral, systemic, or no antibiotics to C57Bl/6 wild type (WT) pregnant mice to determine the impact of oral and systemic antibiotics on the maternal fecal and vaginal mucosal microbial community structure. We will administer vancomycin, doripenem, and neomycin orally in the 7 days prior to delivery or vancomycin, gentamicin, and pipercillin-tazobactum to induce maternal dysbiosis. In

parallel, we will administer amoxicillin orally and parenterally, both commonly prescribed prophylactically to pregnant women near the time of delivery to assess the impact of intrapartum prophylaxis. These mice will allow a unique opportunity to study the effects of antibiotics administered during pregnancy on the fecal and vaginal microbiota of mothers.

1.2.2 Measure the impact of maternal dysbiosis on microbial composition in offspring.

Microbiome studies have consistently shown that the microbiota of neonates is altered by external events such as cesarean delivery, infant diet, and antibiotic administration (Bokulich et al., 2016; Gritz & Bhandari, 2015; Mazzola et al., 2016). In these experiments, we will investigate the effects of antibiotic induced maternal dysbiosis on offspring microbiota composition. Stools and luminal contents of the stomach, ileum, and colon will be collected weekly for sequencing. DNA extraction and amplification will be performed using Eppendorf liquid handling robots. The 16S ribosomal gene (region V4) of each sample will be amplified using 515F and 806R primers, generating paired-end overlapping reads on the Illumina MiSeq platform. Reads will be filtered and binned into operational taxonomic units (OTUs) at 97% similarity. Qiime scripts will be used to classify and align the obtained OTUs. The GreenGenes database will be used to assign taxonomy.

Expected results and alternative plans

The working hypothesis in this aim is that induced maternal dysbiosis alters offspring commensal colonization throughout the developmental period. Our preliminary data suggests that antibiotics administered during pregnancy alters the microbial

communities present at the time of delivery, and that this changes the commensal colonization of offspring from birth through sexual maturity (Fig. 1-3). We expect to confirm these findings and to demonstrate the commonly used prophylactic medications of late gestation alter the maternal microbial delivery environment and the colonization of her offspring. We expect to find that alpha diversity is reduced in both exposed mothers and their offspring, and that microbes belonging to the phyla Bacteriodetes are reduced, while those belonging to Firmicutes and Proteobacteria are enriched. If no differences are discovered in the offspring microbiota between groups, we will administer a 7-day course of orally absorbed antibiotics to the mother during lactation in order directly affect the offspring microbial colonization. As decreased Bacteriodetes:Firmicutes ratio has been linked to obesity in both mice and humans (Bäckhed et al., 2004; Ley et al., 2005), we will weigh the pups weekly throughout the experimental period. We recognize the limitations of 16S rRNA gene analysis when assigning taxonomy, and will therefore perform whole-genome metagenomic analysis on a subset of samples to confirm taxonomy. While we recognize that metagenomic analysis on a subset of samples may reduce the microbial richness and diversity observed, the cost of whole genome sequencing on all samples is prohibitive.

We will sequence the offspring stool and luminal contents of the stomach, ileum, and colon each week to determine the colonization pattern of neonatal mice offspring before and after weaning to solid food. We expect that antibiotic induced dysbiosis will impact the diversity of bacteria in all compartments. Reduced diversity and abnormal colonization during this period of rapid growth and immune establishment has the potential to affect metabolism and immunity (Gomez-Arango et al., 2017; Macpherson,

de Agüero, & Ganal-Vonarburg, 2017). We propose to investigate the functional significance of these alterations in Aim 2. As our preliminary data indicates that offspring of mother's exposed to antibiotics to induce dysbiosis do colonize differently than controls in (Fig. 4), we plan to expand our antibiotic choices to those with both gastrointestinal and systemic absorption. As oral and systemically absorbed antibiotics administered during pregnancy will reach the fetus, we expect that both types of antibiotic exposure will result in reduction in alpha diversity in offspring and an increase in beta diversity between the exposed pups. This will more closely simulate the experience of human mothers and their infants. We do not expect the dysbiotic pups to demonstrate a uniform colonization pattern between mothers, we will, therefore, attempt to correlate observed taxa with observed phenotypes in relation to the proposed challenges to growth and immunity as outlined in Aim 2.

We recognize the differences in murine and human gestation and development. We will therefore attempt to demonstrate similar perturbations to the microbial environment in humans by obtaining a sample of human mothers and their infants. Maternal fecal samples will be obtained before and after antibiotic exposure during delivery. Infant fecal samples will be collected at 2 weeks and at 6 months. Genomic sequencing will be performed after DNA extraction and amplification of the 16S rRNA gene as listed above. Metagenomic sequencing will follow if needed for taxa identity confirmation.

AIM 2: Investigate the functional consequences of dysbiosis during immunological development on offspring growth and resistance to infection.

2.1 Rationale and general design

While the functional consequences of disturbances to the microbiota have yet to be fully explained, early antibiotic exposure has been linked to both obesity and malnutrition (Lange et al., 2016; Tilg & Adolph, 2015), immune deficiency and autoimmunity (Belkaid & Hand, 2014). In a study by Paul et al, prebiotics that altered the bacterial composition of the maternal and neonatal microbiota prevented the transfer of obesity and insulin resistance from pregnant obese rats to their adult offspring, indicating that the microbiota composition during the neonatal period may mitigate the obese and insulin resistant phenotype in adulthood (Paul, Bomhof, Vogel, & Reimer, 2016). De Aguero et al., showed that pups born to germ free mice transiently colonized with bacteria during pregnancy had altered inflammatory responses to infection in comparison to germ free controls. This suggests that altering the microbial milieu at critical periods early in life may change the host's metabolic and immune programming. The working hypothesis of this aim is that altered commensal colonization acquired by offspring after maternal exposure to antibiotics sustains throughout development and impacts metabolic and immune programming, resulting in malnutrition and susceptibility to infection.

2.2 Establish the effect of the altered microbial diversity because of maternal antibiotic exposure on the ileum and liver gene expression profiles before and after the onset of obesity.

Although antibiotics administered to mice and humans during development has been linked to obesity (Azad, Moossavi, Owora, & Sepehri, 2017), the role of antibiotic-induced dysbiosis during gestation has not been well studied. Offspring exposed to low doses of antibiotics during development and throughout life have exhibited decreased hepatic expression of genes involved in cholesterol metabolism and fatty acid synthesis,

and decreased ileal expression of genes involved in Th17 population regulation and antimicrobial peptide production (Cox et al., 2014). In this experiment, whole tissue liver and ileum of 8-week old pups that received antibiotics or no antibiotics during the last week of gestation will be analyzed using RNA-seq to determine differences in gene expression. The distal ileum and liver will be collected and frozen at -80 degrees until RNA is extracted using the RNeasy Mini Kit (Qiagen) as outlined by the manufacturer. RNA-seq libraries will be generated using the TruSeq v3 RNA sample prep (Ilumina). Libraries will be sequenced with 2x75bp paired-end reads in an Illumina Next-seq with an index build from the mm10 reference sequence. Bioinformtatics will be completed by in-house bioinformaticians using TopHat (Johns Hopkins) read mapper.

Next, adult C57Bl/6 offspring exposed or not exposed to antibiotics during gestation will be placed on a western diet (35 kcal% fat (10% saturated), 50 kcal% carbohydrate (15 kcal% from fructose), 15 kcal% protein, and 16 g fiber) upon weaning for 30 weeks. The western diet is designed to emulate the macronutrient consumption in the United States (Wright & Wang, 2010). As obesity has reached epidemic levels in the United States, we expect the mice to develop obesity, but hypothesize that mice exposed to antibiotics during gestation will experience greater obesity, and will suffer more inflammation and poorer immune responses to this common dietary challenge. Glucose tolerance tests and insulin tolerance tests will be performed to assess glucose and insulin sensitivity; and the liver, colon, distal ileum, and mesenteric adipose tissue will be collected every 10 weeks to assess histologic changes. Body weights will be monitored weekly, and liver and adipose tissue weights will be recorded upon collection. Transcriptional responses will be investigated using RNA-seq on the liver and ileum at

30 weeks as outlined above. Stools will be collected, and the 16s rRNA gene analyzed as previously outlined to establish if the changes in response to the western diet differ between the pups colonized at delivery after maternal antibiotic exposure and control mice that experienced normal colonization.

2.3 Establish the effect of the microbial community at the time of weaning on immune programing in response to bacterial endotoxin, abdominal inflammation, and *Citrobacter rodentium* infection.

As bacteria-derived metabolites from the mother have been shown to have an impact on the development of the fetal immune system, the lamina propria and spleen immune cell populations will be examined at weaning (4 weeks) (Gomez de Agüero et al., 2016; van de Pavert et al., 2014). Flow cytometric analysis of lymphoid and myeloid immune cell populations will be completed using the BD LSR Fortessa SORP I Flow Cytometer with BD FACSDiva Software to evaluate the effect of antibiotics administered during gestation on the immune cell populations present in the offspring lamina propria and spleen. As studies have shown that early postnatal colonization influences B cell, iNKT cell, Treg cell, and macrophage differentiation, we hypothesize that reduced alpha diversity associated with maternal antibiotic exposure during gestation will delay the innate and adaptive immune development in offspring exposed to antibiotics during gestation (Fulde & Hornef, 2014).

Next, we will inject lipopolysaccharide, a bacterial endotoxin, or cecal content slurry into the 8-week-old antibiotic-exposed and unexposed offspring's peritoneal cavity to simulate sepsis and peritonitis; or we will gavage *citrobacter rodentium,* a common

murine transient intestinal infection, to examine immune responses to serious systemic, abdominal, or transient infections. Morbidity and mortality scores will be total during sepsis and peritonitis. Weights will be recorded daily during *citrobacter rodentium* infections. Spleen and mesenteric lymph nodes cells will be examined by flow cytometry 24 hours after sepsis and peritonitis induction, and at 4, 8, or 14 days after *C. rodentium* infection to evaluate lymphoid and myeloid cell populations generated during infection. Inflammatory cytokines, such as IL-6, interferon gamma, IL-17, IL-10, TGF-beta, and TNF-alpha, in addition to IgA, will be assayed using the BioLegend mouse multiplex ELISA inflammation panel per manufacturer's instructions to determine functional differences in the inflammatory response. The goal of these studies to detect the functional alterations in host immunity that occur in response to antibiotic-induced dysbiosis during the developmental period by documenting the baseline immune cell populations in newly weaned dysbiotic mice and the ability to fight serious systemic, localized, and transient infections.

Expected results and alternative plans

Previous studies have indicated that the maternal microbial colonization during gestation alone alters innate immune composition in offspring and secondary lymphoid structure development, and that commensals can alter immunity and inflammation (Belkaid & Hand, 2014; Gomez de Agüero et al., 2016). We hypothesize that the maternal dysbiosis in response to antibiotics during gestation will persist throughout the offspring's developmental period resulting in reduced colonization with commensal organisms. These alterations will alter hepatic and ileal architecture and gene expression resulting in downregulated lipid metabolism and delayed immune maturation resulting in

decreased ability to resist serious and transient infection by reducing the repertoire of effector responses to infection. Should antibiotic exposure during gestation not result in prolonged reduction in alpha diversity or increased beta diversity in offspring, we will induce a dysbiotic state in order to test these hypotheses by colonizing the mice with an enteropathic commensal consortium consisting of Bacteroidales and Esherichia coli species that thrive in the intestines under conditions of malnourishment such as obesity or infection (Brown et al., 2015).

Subjects and Settings

C57BL/6 mice will be housed in a specific pathogen free environment in the mouse facilities of the National Cancer Institute in the Fredrick and Bethesda campuses. Mice will be at least 6 weeks old upon mating. Pups will be sacrificed at regular intervals for analysis. IACUC approval will be attained prior to the initiation of experimental procedures.

Power analysis

Power analysis was calculated using G Power and a pilot study containing 20 experimental and 9 control mice. The mean operational taxonomic units (OTUs) of the pilot study experimental group was 202.5 with a standard deviation of 95.8. The mean of the control group was 290 OTUs with a standard deviation of 20.2. Using the Mann Whitney test to detect significant differences between groups with an effect size of 1.7, a power of 95% and a p value less than 0.05, the sample size indicated is 11 per group. Allowing for some attrition because of cannibalization of pups, 15 pups per group will be used.

Variables and Measures

Antibiotics will be orally administered to pregnant mice beginning on the 14th day of gestation, or intraperitoneally administered beginning on the 18th day of gestation and continued until delivery. Stool and alimentary tract organs will be tested throughout development to elucidate differences in microbial colonization between control and antibiotic exposed animals. Biologic metrics will be collected for comparison between treated and untreated groups such as body weight, muscle and adipose tissue weights, glucose tolerance, insulin resistance, and critical organ histology. Blood, spleen, colon, and ileum will be tested for immune cell population differences. When the mice reach maturity, they will be presented with immune challenges to determine immune function differences.

Data Collection Protocol

Fecal samples will be collected from the mothers prior to beginning antibiotics, after antibiotics are discontinued, before weaning, and after weaning. Pup's stool will be sampled before weaning, after weaning, and at regular intervals during immune challenges. Blood, spleen, colon, and distal ileum will be harvested from the pups at regular intervals during development and throughout immune challenge. Body weight, muscle, fat, and organ weights will be collected for comparison. Glucose and insulin tolerance will be tested at regular intervals during diet challenges.

Data Analysis Plan

Bacterial community composition will be determined by amplifying and sequencing the V4 region of 16S rRNA gene from DNA isolated from stool and mucosa. The sequences will be analyzed through bioinformatic pipelines including Qiime. In

addition, metagenomic sequencing of the microbial DNA will be analyzed to derive potential functions of the microbiota at each time point using the PICRUSt pipeline. Innate and adaptive immune cell composition of the lamina propria, mesenteric lymph node, and liver will be assessed using flow cytometry. Numerical differences between groups will be determined using Mann Whitney U and ANOVA with an effect size of at least 80% and a significance level of 0.05. A statistician and bioinformatician will be utilized to assist with data analysis.

Strengths of the proposed study

The overall structure of the immune system in mice and humans is similar, and the mouse has been used as a model for immune function with great success. Using a murine model will allow strict control of genetics, diet, and the environment easing the elimination of confounding variables. In addition, examination of affected tissues is available using animal models as is the ability to follow results longitudinally over the lifespans of several generations. Genetically altered mice are available to test emergent mechanistic hypotheses further strengthening results.

Another strength of this study is the availability of multiple avenues for testing results, and of expert consultants for experimental design and data analysis. Conducting the research in the Immunology and Inflammation program at the NCI allows access to state of the art equipment such as a multi-channel flow cytometer, Illumina sequencer, and robotic DNA extraction. In addition, bioinformaticians, statisticians, biologists, pharmacologists, immunologists, and microbiologists are immediately available consultants. Genetically altered, pathogen free, and germ free mice are available for use in addition to the infrastructure to manage and house them in controlled environments.

Potential Limitations and alternative strategies

Limitations on number of animals utilized for research prevent adequately powered studies to detect small differences in study populations. However, the extreme control of variables available in animal study allows for reduced numbers needed to detect significant differences. A pilot study indicates that differences in the microbial populations are significant even using a small number of animals. Human studies have also documented significant differences in the microbiota of antibiotic exposed infants after delivery.

Despite the mouse's long history as a model for human disease, there are differences between human and murine immunology. The mouse immune system is skewed toward the lymphoid population, while the human has a higher neutrophil population. Other differences exist in both innate and adaptive immunity, which may impair translation to the human. Results from these trials will need to consider the mechanisms of immune function in both the murine and human systems to infer translation.

Timeline

Year 1- Develop model of murine antibiotic administration to pregnant mice. Determine impact of oral and systemic antibiotics on murine fecal microbiota throughout development. Document alpha and beta diversity differences, and taxonomic colonization differences between antibiotic exposed and control mouse pups before weaning, after weaning, and at maturity.

Year 2- Investigate the consequences on murine growth and immune development of altered microbial colonization by comparing growth patterns and immune cell populations between antibiotic exposed and control mouse pups.

Year 3- Investigate the functional consequences of dysbiosis by challenging antibiotic exposed mice with commonly occurring immune challenges encountered in a Western population. Complete analyses. Manuscript preparation

Perinatal Antibiotic Prophylaxis—Friend or Foe?

Neonatal sepsis and maternal surgical site infections remain a significant source of morbidity. In the United States 4.5% of neonatal deaths result from sepsis in the first month of life (Unicef Child Mortality Estimates, 2018). Group B streptococcus (GBS, *Streptococcus agalactiae*) remains the foremost causal organism of morbidity in neonates with *Escherichia coli* (*E. coli*) recently emerging as the 2nd most common pathogen to cause neonatal disease (Shah and Padbury 2014). Together they account for 70% of neonatal early onset sepsis (Simonsen et al. 2014). CDC guidelines recommend chemoprophylaxis for all women with GBS colonization and for those with other risk factors for infection such as preterm labor, prolonged rupture of membranes, or maternal fever (Verani et al. 2010). Though antibiotic prophylaxis has reduced the incidence of early onset GBS sepsis nearly 80% to the current levels, late onset (>72 hours after birth) sepsis incidence remains unaffected and other pathogens, such as *E. coli*, have increased (Bauserman et al. 2013). Maternal wound infection complicates 2-7% of cesarean sections (Kawakita and Landy 2017). The American College of Obstetricians and Gynecologists recommends antibiotic prophylaxis for all cesarean deliveries and the addition of a second antibiotic when the cesarean is not elective (Committee on Practice Bulletins-Obstetrics 2018). Healthy women could, therefore, receive 3 or more doses of parenteral antibiotics prior to delivery.

Although intrapartum antibiotic prophylaxis (IAP) decreases morbidity in both mothers and their infants, the number needed treat early-onset GBS disease is 1191 (Angstetra et al. 2007). A Cochrane review of 4 trials including 852 women in 2014

indicated that although IAP did reduce the incidence of early onset GBS infection, it did not reduce mortality from GBS infection, all-cause mortality, or mortality from other bacterial infections (Ohlsson and Shah 2014). A similar Cochrane review examined 95 trials involving 15,000 women on the effect of surgical prophylaxis and found that the risk of wound and womb infections were reduced by 60-70% (Smaill and Grivell 2014). This statistic held true whether the antibiotic was given before or after cord clamping, and whether the cesarean section was elective or not (Smaill and Grivell 2014). It appears that therapies to prevent GBS disease could be more targeted and that surgical prophylaxis, if effective after cord clamping, may not need to reach the infant at all.

Several studies in humans and farm animals indicate that long term or repeated intermittent doses of oral antibiotics impacts the growth and obesity (Turta and Rautava 2016; Feighner and Dashkevicz 1987). Fewer studies have addressed the impact of antibiotics given for short duration, or injected antibiotics. None that we are aware of have addressed the long-term impact on growth or obesity of limited doses of injected antibiotics, like those given to women prior to delivery. In this study we administer antibiotics to pregnant C57Bl/6 mice during the final days of their gestation. We examine the effects on the maternal and offspring microbiota until weaning. We then place the offspring on regular chow or on a western style diet that is high in saturated fat and fructose and low in fiber in order to emulate the macronutrient consumption in the United States as documented by the CDC in 2010. Offspring are then monitored until they are 30 weeks old for changes in fecal microbiota, growth, obesity, glucose tolerance, insulin levels, and histologic and gene expression changes within the intestinal tract and liver.

Animal research allows us the strict controls needed to discern how changes to the microbiota effect the host organism. Considering that bacteria outnumber host nucleated cells by approximately 10:1 (Sender et al. 2016), and that bacterial colonization is influenced by genetics, diet, medications, and environment (Daniel et al. 2014; Kurilshikov et al. 2017; David et al. 2014; Lankelma et al. 2017), it is difficult in human studies to observe experimental variation amidst the noise of normal human variation including cultural, social, genetic, and dietary differences. In addition, mechanistic studies are possible in animals. This study controls for genetics, environment, and diet by using genetically identical littermates, maintaining them in a specific pathogen free environment, and by autoclaving all food and water.

Bedside nurses, being the closest healthcare providers to the patient, are in a unique position to influence the microbiota. They are responsible for the patient's immediate environment, diet and feeding practices, hygiene, wound care, intravenous access, indwelling intravenous and urinary catheter care, visitor access, and visitor hygiene. Nurse practitioners take on the additional responsibilities of prescribing medications, including antibiotics, choosing diets and safe feeding routes, inserting invasive catheters and performing invasive procedures. It is important to understand the role of the microbiota in human health so that decisions regarding all of these aspects of care are well informed.

Impact of Intrapartum Antibiotic Prophylaxis on Offspring Microbiota—A Review

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Abstract

Infants are born into a world filled with microbes and must adapt to their presence without undue immune response while exploiting their ability to produce otherwise unavailable nutrients. The process by which humans and microbes establish this relationship has only recently begun to be studied with the aid of genomic methods. Nearly half of all pregnant women receive antibiotics during gestation in order to prevent maternal and neonatal infection. Though this has been largely successful in reducing

early onset sepsis, we have yet to understand if there are long-term consequences to developing infants that would not go on to develop sepsis. Studies involving antibiotic use in infants suggest that dysbiosis during this period is associated with increased obesity, allergy, autoimmunity, and chronic diseases in adulthood, but research around the limited doses of intravenous antibiotics used for peripartum prophylaxis is limited. In this review, we focused on the state of the science regarding the newborn microbial colonization process and the effects of peripartum antibiotic prophylaxis. We found that though there is wide variety in the specific bacteria that colonize infants from delivery, limited parenteral antibiotic administration prior to delivery consistently affects the microbiota of infants by decreasing bacteria in the phylum *Bacteriodetes* and increasing those in the phylum *Proteobacteria*, thus altering the normal pattern of colonization that infants experience. Delivery by cesarean section and formula feeding enhance and prolong this effect. The impact this change in early colonization has on growth or immune programming in the developing offspring has not been well studied in humans or animals.

Background

As microbes evolved more than a billion years before even the first eukaryotes and outnumber all eukaryotic species by an estimated 10^{23} times, it is safe to say that every being on earth has evolved in their presence and formed either a symbiotic or competitive relationship with them in order to survive. Often this relationship changes depending on the micro and macro-environments in which each individual species inhabits. Although there is now some evidence that bacteria or at least bacterial products are present in the wombs of humans, a human infant is born nearly, if not entirely, sterile

(Perez-Muñoz et al., 2017). By 4 weeks of age, the infant is colonized with 4.4 x 10^{12} bacteria, and this number will increase to a total of 3.8-4.4 x 10^{13} by adulthood (Sender et al., 2016). These bacteria are distributed on every surface interface, outnumbering human nucleated cells by roughly 10:1. Interestingly, some bacterial lineages have coevolved in concert with humans, speciating as humans diverged from ancient hominids (Moeller et al., 2016). Thus, bacterial niche specialists are transferred longitudinally from mother to child exploiting the unique characteristics of their preferred habitat. Bacterially-derived products for growth and development are then available to the new host and for bacterial opportunists leading to a robust environment that competes and adapts to changes infant progresses toward a more stable adult-like microbiota.

Infant intestinal colonization proceeds typically with early colonizers representing diverse facultative anaerobes and then increasing in strict anaerobes as the infant gut proceeds toward maturity (Ferretti et al., 2018). It takes nearly 3 years for the human infant gut to resemble the adult colonization pattern. Though the initial bacterial inoculum occurs at delivery through maternal vaginal, fecal, skin and environmental exposure, the microbiota of identical twins is no more similar than that of fraternal twins (Yatsunenko et al., 2012). An individual's microbiota resembles the microbial ecology of those living in close proximity more than those living separately. This supports the notion that bacterial colonization is not genetically imposed, but opportunistic and proceeds differently depending on local niche conditions. Species that initially colonize the infant intestinal tract originate from the mother and the environment in equal portions, whereas the oral microbiota is shared almost entirely with the mother during the first several days of life (Ferretti et al., 2018). These early colonizers are species of low abundance in the

maternal biome and are transient as infants settle into distinct infant colonization patterns that only gradually resolve into stable adult microbiotas. This is likely a result of environmental differences in the infant such as the increased pH of infant body cavities and exclusive milk feeding. Still, the infant microbiota remains more similar to the mother than to other adults, especially in the intestinal tract where maternal strains seem to have an ecologic advantage and remain stable over time (Ferretti et al., 2018). The microbiota that inhabit infants is distinct from the adult microbiota, however, and performs specific functions that change as the infant matures (Yatsunenko et al., 2012). Thus, the infant microbiota is seeded with some stable species from the mother and expands over time with a series of microbes present locally and adapted to infant-specific microenvironments. These pioneering microbes colonize the infant in a predictable pattern and are poised to impact the developing host before settling into niche-specific adult colonization patterns within the first few years of life.

Although the maternal microbiota is strongly linked to her infant's, the duration of pregnancy, delivery mode, feeding practices, and antibiotic exposure all influence the microbial colonization of infants during this critical developmental period (Dogra et al., 2015; Bokulich et al., 2016). Epidemiological studies suggest an increased risk of childhood asthma, obesity, allergy, and inflammatory bowel diseases associated with delivery by cesarean section (Papathoma et al., 2016; Wu et al., 2016; Mueller et al., 2015; Li et al., 2014). Whether these associations are the result of delivery mode alone or in combination with antibiotic use has not been determined because the vast majority of cesarean section patients receive antibiotic prophylaxis before delivery.

Recommendations for Antibiotic Prophylaxis at Delivery

Surgical prophylaxis affects 32% of all U.S births and reduces the incidence of post-surgical infection by 60-70% (Smaill and Grivell, 2014; Hamilton et al., 2015). The American College of Obstetricians and Gynecologists (ACOG) recommends that all cesarean deliveries be preceded by antibiotic prophylaxis administered within an hour of delivery. Addition of a second antibiotic should be considered if the cesarean is non-elective. Vaginal cleansing with povidone-iodine or low-alcohol chlorhexidine gluconate should be considered before cesarean if the woman is in labor or has ruptured membranes. For patients with preterm rupture of membranes less than 35 weeks of gestation, a 2 day course of intravenous ampicillin and erythromycin followed by a 5 day course of oral amoxicillin and erythromycin is recommended (Committee on Practice Bulletins-Obstetrics, 2018).

Streptococcus agalactiae (Group B Streptococci, or GBS) remains the leading cause of infectious morbidity and mortality in newborns. Intrapartum antibiotic prophylaxis (IAP) administered to all GBS positive women has resulted in an 80% reduction in early onset neonatal sepsis (Verani et al., 2010). The Centers for Disease Control and Prevention (CDC) recommends that all pregnant women be tested by vaginal-rectal culture within 5 weeks of delivery, and that GBS positive women be treated for at least 4 hours prior to delivery. Women in preterm labor with unknown GBS results should begin antibiotics for latency or prophylaxis and continue them for 48 hours until negative culture results are obtained. In addition, IAP should be administered to any woman with a previous infant with invasive GBS disease, bacteriuria during any trimester, rupture of membranes greater than 18 hours, or intrapartum fever greater than

38.0° C (100.4°F) (Committee Opinion No. 485: Prevention of Early-Onset Group B Streptococcal Disease in Newborns: Correction., 2018). As a result of these guidelines, up to 50% of all pregnant women receive IAP (Money et al., 2013; Smaill and Grivell, 2014).

The advent of genomic analysis has led to the recognition that environmental factors influence gene expression and therefore impact human development, function, and behavior. One such environmental factor is the microbial milieu that influences infant colonization during early life. The postnatal colonization process potentially affects programming of epithelial barrier function, gut and immune homeostasis, neurobehavioral development, and angiogenesis (Diaz Heijtz, 2016). Antimicrobials administered to the mother during pregnancy may alter the maternal and infant microbiota initiating long-term effects on infant immunity, metabolism, and behavior. To date research has largely focused health effects in children and adults resulting from microbiota perturbations as a consequence of ingested antibiotic administration during early childhood. While studying the direct effects of antimicrobial administration is important, establishing the impact of perinatal antibiotic prophylaxis, which is largely administered intravenously and of limited duration, will elucidate the effects on maternal and infant colonization, and the resultant impact on metabolic, immune, and neurobehavioral programing. This review will focus on research that specifically addresses the impact of peripartum antibiotic prophylaxis on infant microbial colonization.

Methods

A literature search was conducted in PubMed, OVID Medline, Web of Science, and EMBASE to find original research that addressed the impact of intrapartum antibiotic prophylaxis (IAP) on infant microbiota and outcomes. The search terms from all databases were ((“anti-bacterial agents" OR "antibiotics”) AND ("microbiota" OR "microbiome") AND ("infant"). Medical Subject Headings (MeSH) terms were used to encompass all terminologies related to these core terms. All databases were searched for original research articles published in English within the past 10 years. Authors, abstracts, and citations were uploaded into Covidence for a two reviewer (CD and SP) screening. Systematic literature reviews were screened, but not included. Inclusion criteria for the search included, 1) original research, 2) examination of healthy term pregnancies, 3) experimental design that included a control group of infants/offspring that were delivered vaginally without IAP and 4) animal studies were also included if the purpose was to address the microbial effects of antibiotic administration under controlled conditions and there was an appropriate control group of genetically normal animals. Exclusion criteria includes, 1) administration of antibiotics to infant and not mother, 2) infants included in the study are premature or not healthy (i.e. necrotizing enterocolitis, Cystic Fibrosis). Articles that were included in the review are summarized in **Table 1**.

Maternal microbiota and Intrapartum Antibiotic Prophylaxis

Studies of the vaginal microbiota reveal that while there is no single representative vaginal microbial community, there are several common consortiums that have been labeled as “core microbiomes” (Ravel et al., 2011). These consortiums tend to remain stable over time in each individual with occasional intermittent transitions to other

community states depending on women's age, health, ovarian cycle, sexual activity, and reproductive state. Most of the core microbiome consortiums, or community state types (CST) are dominated by *Lactobacillus* spp. in humans, especially in Caucasian and Asian women, while Hispanic and Black women tend to have more diverse vaginal microbiotas (Ravel et al., 2011). During pregnancy *Lactobacillus* species are favored, however, and CSTs dominated by other bacteria are rarely present, even in non-Caucasian women (Walther-António et al., 2014; Romero et al., 2014). Although CSTs dominated by species other than *Lactobacillus* may be more common in asymptomatic non-Caucasian women, they are more likely to be associated with increased risk of bacterial vaginosis, adverse perinatal outcomes, and other infectious conditions (Romero et al., 2014; DiGiulio et al., 2015). It is likely that the acid-producing *Lactobacillus* species are selected during pregnancy for their pH-lowering properties that reduce colonization with more pathogenic bacterial species. The mechanism for bacterial selection in the special condition of pregnancy has not been elucidated to date, but is speculatively driven by elevated estrogen leading to the accumulation of glycogen in the maturing vaginal epithelium (Roesch et al., 2017).

GBS was identified in 1935, recognized as the leading cause of early onset neonatal sepsis in the 1960s, and identified as the most common cause of neonatal sepsis and meningitis in developed countries by the 1980s (Le Doare and Heath, 2013). Approximately 30% of women are colonized with GBS, and 50% of these will transmit the bacteria to their infants, 1% of whom will develop invasive disease. Early onset GBS-associated morbidity can be acquired by the fetus via vertical transmission and present as pneumonia, sepsis, or meningitis at delivery or within the first few days of life. Late onset

GBS disease is usually acquired perinatally or environmentally and usually presents as meningitis. GBS disease after 90 days of age is rare (Le Doare and Heath, 2013). The introduction of IAP for GBS has reduced the incidence of early onset GBS morbidity and mortality from 1 in 200 births to 1 in 4000 births (GBS | Fast Facts and Statistics | Group B Strep | CDC). IAP has no effect, however, on late-onset GBS disease (Schrag and Verani, 2013).

As IAP is administered in vaginal deliveries for the prevention of GBS disease, most studies that examine the effect of IAP on the infant microbiota are examining the covariance of both antibiotic exposure and GBS colonization. Studies have shown that though IAP does reduce neonatal early-onset sepsis (EOS), it does not prevent maternal transmission of GBS, or late-onset GBS disease (Toyofuku et al., 2017; Roesch et al., 2017; Patras and Nizet, 2018). In addition, GBS is found in culture-negative mothers, though in low abundance, when using culture-independent methods to examine maternal samples (Toyofuku et al., 2017; Roesch et al., 2017). In non-pregnant women, GBS has been shown to occur in all CSTs and is associated with certain species, such as *Prevotella bivia,* within the vaginal environment (Rosen et al., 2017). In pregnant women, GBS was also present in all CSTs, but in higher abundance in CSTs deficient in *Lactobacillus.* Low *Lactobacillus* CSTs were also inversely correlated with gestational age at delivery leading to the speculation that *Lactobacillus* may be protective against preterm delivery (DiGiulio et al., 2015). As preterm infants have higher incidence and mortality from GBS EOS, preterm labor is an important indication for targeted GBS prophylaxis. Severe GBS infection was associated with loss of vaginal bacterial diversity as a result of the

abnormal dominance of this taxa, so bacterial load may be of significance if using a targeted prophylactic treatment strategy (Roesch et al., 2017).

IAP targets gram positive species in the case of GBS prophylaxis and both gram-positive and gram-negative species in the surgical setting. Consequently, commensal gram-positive bacteria, such as the dominant vaginal *Lactobacillus* spp., are drastically reduced after IAP (Roesch et al., 2017). Gram positive gastrointestinal (GI) species such as *Bacteriodes* and *Bifidobacteia* are adversely affected as well (Aloisio et al., 2014; Nyangahu et al., 2018). With the reduction of these keystone species, opportunistic bacteria become the early colonizers and maternal vaginal bacterial communities have higher diversity after IAP (Roesch et al., 2017; Cassidy-Bushrow et al., 2016). Gomez-Arango et al. found that infants colonized more similarly to their mothers, and with less bacterial diversity, if their mothers had not received IAP.

Infant microbiota

How infants become colonized is a subject of intense study. Though great variety occurs within and between individuals, metabolic pathways are stable, suggesting that local bacteria colonize the human body by exploiting and competing for the resources available in different niches (Human Microbiome Project Consortium, 2012). Colonization presents an enormous challenge to the newborn as it emerges from a protected womb to encounter bacterial, viral, and fungal antigens in their millions in addition to food antigens in milk, and immunogenic antigens in vaccines administered shortly after delivery. While amniotic fluid and colostrum favor immune tolerance toward maternal and bacterial antigens, breastmilk exerts a strong selective influence on the early

microbiota (Brugman et al., 2015). Early breast milk and colostrum contain not only lipids, protein, lactose, and oligosaccharides for nutrition, but large amounts of IgA, immune cells, cytokines, hormones, growth factors, and non-specific immune factors such as lysozyme and lactoferrin (MohanKumar et al., 2017; Donnet-Hughes et al., 2010; Goldman, 1993).

Infant fecal colonization proceeds in an orderly fashion as the GI tract changes from an anaerobic and nearly sterile environment in utero to an aerobic environment with the first breathing and swallowing of air. This reverts to an anaerobic environment again at the host-bacterial interface within the first few days after the introduction of breastmilk, the passage of the first stools, and the metabolic activities of the early colonizers (Jost et al., 2012; Tourneur and Chassin, 2013). IAP may influence not only the initial seeding of the infant from the mother at delivery by altering her vaginal, skin, and anal microbiotas, but also by exerting antimicrobial properties directly through the breastmilk on the infant's oral cavity and GI tract.

Nearly every study reviewed reported changes to the infant fecal microbiota after maternal IAP. Decreased Bacteroidetes and Actinobacteria and increased Proteobacteria were common (Aloisio et al., 2016; Mazzola et al., 2016; Nogacka et al., 2017; Azad et al., 2016; Nyangahu et al., 2018). See **Table 2** for a summary of infant bacterial colonization differences after IAP in selected studies. These results were seen after IAP in both vaginal and cesarean section deliveries. In mice and in infants whose mothers were known to be colonized with GBS, offspring had higher *clostridia* and *enterococcal* colonization after IAP (Cassidy-Bushrow et al., 2016; Aloisio et al., 2014). Increases in these bacteria may be the result of the reduced Lactobacillus colonization seen in both

animals and in humans with GBS colonization (Roesch et al., 2017; De Gregorio et al., 2015; Vrbanac et al., 2018).

As the neonatal GI tract experiences transient colonization with facultative anaerobes before assuming stable colonies of strict anaerobes, the question arises whether IAP affects early colonizers only, or whether the effects are maintained throughout these early transitions in the neonatal GI tract. Corvaglia et al. concentrated on selected bacterial species by performing qPCR on infant fecal samples, and found that IAP resulted in lower *Bifidobacterial* counts at 7 days, but were no different at 30 days of life, though they reported decreased counts if the infants were not exclusively breast fed (Corvaglia et al., 2016). Other studies that examined total bacterial diversity in mice and humans using 16S rDNA sequencing found altered colonization patterns lasting from 3 months to a year (Mazzola et al., 2016; Nogacka et al., 2017; Azad et al., 2016; Miyoshi et al., 2017). The combination of cesarean section and IAP or the combination of formula feeding and IAP extended the effect size and the duration of altered microbial colonization patterns (Yasmin et al., 2017; Corvaglia et al., 2016; Nogacka et al., 2017; Mazzola et al., 2016).

Cesarean section delivery has been linked to increased risk of allergy, asthma, obesity, autoimmune diseases, and late life chronic illnesses such as inflammatory bowel disease (Bager et al., 2012; Mueller et al., 2015; Papathoma et al., 2016; Black et al., 2015). It has not been determined whether these increased risks are the result of the obstetric problems that lead to cesarean delivery, the antibiotics that precede delivery, or the result of altered microbial colonization from missed vaginal exposure. Azad et al. attempted to answer this question by comparing infants grouped by delivery method and

IAP. They found that all infants exposed to IAP, regardless of delivery method, had reduced fecal *Bacteroidetes* and increased *Proteobacteria* at 3 months, but that those delivered by cesarean section had a larger effect size that persisted for a longer duration. Infants delivered by emergent cesarean section, and therefore exposed to obstetric problems and more antibiotics, had dysbiosis that persisted up to a year. Dominguez-Bello et al. found differences in the infant microbiota based on delivery methods and demonstrated that infant colonization could be influenced by exposure to vaginal fluids administered postnatally after cesarean section. They also found no differences in maternal vaginal colonization after IAP (Dominguez-Bello et al., 2016). This agrees with Nyangahu et al. that found no difference in vaginal colonization in mice 4 days after treatment with antepartum oral vancomycin. Both groups stress that their cohorts were small and perhaps not powered to detect changes in the vaginal microbiota. Large differences did exist, however, in the bacterial colonization and immunoglobulins in offspring stomachs, a proxy for mouse breastmilk, supporting the notion that it is the breastmilk that is most influential in shaping the gastrointestinal microbiota of offspring after delivery (Nyangahu et al., 2018). Stokholm et al. conducted a study of 738 pregnant women at 36 weeks of gestation and found changes in the vaginal microbiota after oral antibiotic exposure, but these were orally administered antibiotics prescribed for longer duration than IAP and secondary to infection (Stokholm et al., 2014).

The gastrointestinal microbiota has been consistently shown to exert major influences on obesity and immune system development and function (Yu et al., 2018; Graham et al., 2015). Antibiotics administered during infancy in mice and humans increase susceptibility to allergy, asthma, and obesity, but most studies tested orally

administered antibiotics administered in repeated doses similar to childhood therapy for infection, or in small doses over long duration as in exposure to antibiotics in meat or water (Turta and Rautava, 2016; Cox et al., 2014; Rasmussen et al., 2018; Russell et al., 2012). Few studies have investigated the outcomes associated with maternal peripartum antibiotic exposure, which is characterized by intravenous administration of short duration.

To understand the impact of perturbations to the microbiota during gestation versus disruptions during adulthood as a result of parenterally administered antibiotics, Munyaka et al., injected C57Bl/6 wild type mouse dams for 7 days prior to delivery with cefazolin, a 1st generation cephalosporin commonly used for cesarean prophylaxis, and then exposed them to dextran sulfate sodium (DSS) at 7 weeks of age. Offspring of dams exposed to antepartum antibiotics developed earlier and more severe colitis supporting the theory that injected antenatal antibiotics can exert long-term effects on offspring GI tract immunity (Munyaka et al., 2015). Miyoshi et al. treated IL-10 deficient mice neonatal and adult mice with a 4-week course of oral vancomycin, then exposed them to DSS. They found that bacterially colonized, but not germ-free offspring, exposed to antibiotics during the pre-weaning period developed persistent dysbiosis that lasted into adulthood and increased susceptibility to spontaneous and induced colitis. Early dysbiosis provoked inflammatory T cell programming and decreased regulatory T cells and anti-inflammatory mediators in the lamina propria and mesenteric lymph nodes of mice genetically predisposed to colitis (Miyoshi et al., 2017). Germ free and adult mice exposed to antibiotics did not develop colitis, revealing that it was perturbation during early colonization that drove changes in immune programming. The same T cell

programming changes occurred when non-obese diabetic mice were exposed to short courses of oral antibiotics during gestation and trended toward increased type II diabetes incidence (Tormo-Badia et al., 2014). In addition, neonatal mice exposed antibiotics during gestation produced inflammatory T cells that were unable to sustain normal interferon gamma production when stimulated (Gonzalez-Perez et al., 2016). These results indicate the microbiota derived products are required during early colonization and immune development in order to influence proper immune cell lineage programming and for intact signaling to elicit normal function.

The only study that focused on long-term outcomes in human infants after in-utero exposure to antibiotics determined after examining the records of 97,000 children, 336 of which had developed type I diabetes, that in-utero antibiotic use of any type or duration was not associated with childhood onset type I diabetes (Haupt-Jorgensen et al., 2018). Long-term outcomes associated with intermittent therapeutic antibiotic courses during childhood or prolonged subtherapeutic courses as in meat or water consumption have been linked to many allergic, autoimmune, and inflammatory processes as discussed earlier, but few studies have addressed long-term outcomes after brief exposure to intravenous doses as seen in IAP.

Conclusions

Throughout evolutionary history, infants have had to cope with a barrage of microbes shortly after if not before delivery. As human anatomy has evolved to maintain the maternal anus within close proximity to the vagina despite the risk of maternal infection from gastrointestinal microbes, we can surmise that the GI microbiota is important to the overall health of the developing infant. That certain microbes have

evolved alongside humans as they emerged from the ancient hominids speaks to the symbiotic nature of the relationship. However, our multifaceted and conserved immune system points to the need for tight control in order to exploit the benefits of commensalism without incurring harm to the host from either too much or too little immune response.

Since the advent of IAP, culture proven early onset GBS disease has declined by 68%, though decreases are not the same across all groups in the United States. Black and preterm infants continue to have a higher incidence, late onset GBS disease is unaffected, and adult onset invasive GBS disease has continued to increase substantially (Phares et al., 2008). A Cochrane Review in 2014 reported a 60-70% decrease in serious maternal post-partum infection after cesarean section when prophylactic antibiotics are administered (Smaill and Grivell, 2014). Clearly controlling the invasion of pathogenic microbes is an important part of preventative medicine for pregnant women and their infants. Until technology allows selected bacteria to be eliminated or disarmed, prophylaxis is the best strategy to reduce the risk of invasion. However, as with any medical intervention, risks and benefits must be weighed, and we currently do not know the extent of the risks involved in the prophylactic administration of antibiotics that are intravenously administered for limited duration surrounding delivery.

The most obvious first steps include limiting cesarean sections to cases where obstetrical problems warrant it and using molecular methods to detect GBS in the delivery room and targeting prophylaxis to heavy colonizers. Vaccine development has become a priority and in 2014 the World Health Organization began consulting on GBS vaccine development and focusing on maternal immunization (Lin et al., 2018). In

addition, research must continue to determine the impact of intravenous antibiotic administration of limited duration on the maternal microbial milieu and the colonization process of offspring. Most studies to date have focused on oral administration of antibiotics during infancy. Understanding the importance of exposure to vaginal and anal microbes during delivery; whether environmental, maternal skin, or breast milk exposure drive infant colonization; if certain microbes can be restored after antibiotic exposure; how microbes interact within the different body niches to impact the long-term growth and immunity of offspring. Answers to these questions will help determine strategies to prevent disease without negatively impacting the benefits of our long history with microbes.

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Table 1. Selected studies examining perinatal antibiotics and offspring microbiota.

Author and Year	Study Design and Source	N	Type of Microbiome Sample	Results
Keski-Nisula et al., 2012	Observational, human.	45 mother-baby dyads	Maternal: vaginal Neonatal: oral	*Lactobacillus* transfer from mother to infant delayed in IAP infants.
Tormo-Badia et al., 2013	Experimental, mice.	10 mice	Fecal	Frequency of CD3+ and CD8+ T cells in the mesenteric lymph nodes were significantly higher compared with offspring.
Aloisio et al., 2014	Observational, human.	52 newborns-vaginally delivered	Fecal	*Escherichia coli*, *Bacteroides fragilis*, and *Bifidobacterium* were most abundant in the control group while *Lactobacilli* and *Clostridium difficile* were much lower. IAP infants had decreased *Bifidobacterium* and *Escherichia coli*.
Munyaka et al., 2015	Experimental, C57Bl/6 WT mice.	Four mice	Fecal	Fecal bacterial composition was altered at seven weeks. Including decreased richness and modified functional pathways. Increased *Clostridium* in antibiotic group.
Aloisio et al., 2016	Observational, human.	20 term infants	Fecal	Decreased *Actinobacteria*, *Bacteroidetes* in the IAP group and increased *Proteobacteria* in the IAP group. Higher abundance of gram-negative phyla in IAP group.
Azad, et al., 2016	Prospective longitudinal, human.	198 infants	Fecal	IAP infants deficient in *Bacteroidetes*. *Firmicutes* and *Proteobacteria* higher in IAP group. Genus *Parabacteroides* and *Bacteroides* underrepresented at three months in all IAP categories. Differences persistent for one year for emergent cesarean delivery only.
Cassidy-Bushrow et al., 2016	Observational, human.	262 infants	Fecal	If IAP, no GBS associated infant gut microbiota at one or six months. If no IAP, GBS was associated with distinct microbiota at six months.
Corvaglia et al., 2016	Longitudinal, human.	84 mother-infant dyads	Fecal	*Bifidobacteria* count lower in IAP group at 7 days, no difference at 30 days. Higher

				incidence of *Bifidobacteria* and *Lactobacillus* in both groups if exclusively human milk-fed.
Dominguez-Bello et al., 2016	Longitudinal, human.	18 mother baby dyads	Fecal	Vaginal seeding partially restores microbiota to similarity to maternal vaginal microbiota. No differences in vaginal samples based on antibiotics (2 vaginal and all C/S received IAP).
Mazzola et al., 2016	Observational, human.	26 infants	Fecal	Lover diversity in BF-IAP vs BF-Controls. *Actinobacteria* in BF-IAP infants and absent from controls. BF-IAP dominated by *Enterobacteriacea* family mostly *Escherichia*. *Bifidobacteria* not detected in IAP infants.
Gomez-Arango et al., 2017	Observational, human.	36 mother-baby dyads	Placental, oral and fecal.	Infant microbial profiles less similar to maternal profiles if exposed to IAP. *Proteobacteria* more abundant after IAP, whole *Streptococcaceae*, *Gemillaceae*, and *Lactobacillus* dominated in no IAP groups.
Gonzalez-Perez & Lamouse-Smith, 2017	Experimental, C57Bl/6 mouse.	15 offspring mice.	Outcomes only	Offspring of antibiotic treated dams have fewer cytokine responses in CD8+ T cells and altered T cell receptor signaling
Miyoshi et al., 2017	Experimental, mice.	5 WT dams and 2 offspring litters per dam	Fecal	Genetically susceptible treated offspring were more susceptible to subclinical inflammation and DSS-induced colitis. Inflammatory mRNA levels are elevated in exposed mice.
Nogacka et al., 2017	Prospective longitudinal, human.	40 vaginal delivered infants	Fecal	IAP reduced levels of *Bacteroidetes* in exclusively breast-fed infants, but not formula fed infants. *Proteobacteria* and *Firmicutes* were higher in IAP exposed infants both breast and formula fed. Effect of IAP remains for at least the first months of life.
Roesch et al., 2017	Observational, human.	18 mothers	Vaginal	Higher microbial diversity in IAP groups, *Lactobacillus* greatly reduced in PCN treatment, *Pseudomonas* increased with PCN treatment.

Stearns et al., 2017	Observational, longitudinal, human.	74 infants	Fecal	Decreased *Bacteroides*, increased *Proteobacteria* in IAP groups, longer duration of ABX longer effect on microbiota.
Haupt-Jorgensen et al., 2018	Epidemiological, human.	96,840-Danish infants/children.	N/A	In utero antibiotics approaches significance for protecting against Type I diabetes. Early stimulation of B-cells reduces the incidence of autoimmune diabetes.
Imoto et al., 2018	Cross-sectional, human.	33 infants	Fecal	Beta diversity significantly different between antimicrobial use at delivery. Not significant by delivery mode. *Bifidobacteria* were less abundant in infants exposed to maternal antibiotics.
Nyangahu et al., 2018	Intervention, BALB/c mice.		Fecal	Decreased alpha diversity in all treated groups. Distinct community if during gestation vs postpartum. Treated had higher proteobacteria, lower *Bacteriodetes*.

Note. IAP= intrapartum antibiotic prophylaxis; GBS= Group B streptococcus; WT= wild type; DSS= dextran sulfate sodium.

Table 2: Comparison of gut microbial changes at the phyla level with antibiotic administration in humans.

Phylum	Aloisio et al., 2014	Cassidy-Bushrow et al., 2016*	Azad et al., 2016	Nogacka et al., 2017@	Mazzola et al., 2016	Corvaglia et al., 2016^	Aloisio et al., 2016	Gomez-Arango et al., 2017	Imoto, 2018
Actinobacteria#	-				-	-	-		-
Bacteroidetes	-		-	-	-		-		
Firmicutes		+	+	+	+				
Proteobacteria	-		+	+	+		+	+	

#*Bifidobacterium* genus across all studies. *Specifically studied antibiotic administration with GBS colonization. Infant's whose mothers were GBS positive were enriched in *Firmicutes*. @Reduced levels of *Bacteroidetes* in exclusively breast-fed infants, but not formula fed infants. ^Decrease in *Bifidobacterium* doesn't persist to 30-day collection, only at 7 days and is influenced by incidence of breastfeeding.

Using a Murine Model to Explore the Impact of Antenatal Antibiotics on Offspring Microbiota and Obesity Outcomes

The purpose of this study is to determine the effects of perinatal antibiotic administration on the microbiota of offspring and the results of altered microbial colonization on growth and metabolism. A murine model to study the effects of perinatal antibiotics on offspring was chosen because confounding variables can be controlled to a much greater extent, tissues are available for analysis, and the short life cycle of the experimental subjects allows for robust longitudinal study. The following document describes the methods by which the experimental hypotheses were addressed, the procedures followed to minimize confounding results, and the measures by which collected data were analyzed.

Experimental Hypotheses

The advent of genomic analysis has led to the recognition that environmental factors influence gene expression and therefore impact human development, function, and behavior (Diaz Heijtz 2016; Wang et al. 2016; Gensollen et al. 2016). One such environmental factor is the microbial milieu that colonizes the infant during early life. The postnatal colonization process effects the developmental programming of epithelial barrier function, gut and immune homeostasis, and angiogenesis (Diaz Heijtz 2016). Antimicrobials administered to the mother during pregnancy, therefore, may initiate long-term effects on infant immunity, metabolism, and behavior. To date research has largely focused on the role of microbiota perturbations as a result of ingested antibiotic administration directly to infants during their developmental period. We questioned

whether the limited doses of injected antimicrobials that are administered to females prior to delivery were present long enough to have an effect on the microbial environment of the maternal vaginal or gastrointestinal tract.

Hypothesis 1: Injected antibiotics administered in the last days of pregnancy alter the maternal vaginal and gastrointestinal bacterial colonization at the time of delivery. See Figure 1 for the experimental protocol to address hypothesis 1.

Next, we considered whether alterations to the maternal microbial environment in the last days of pregnancy would translate to the changes in the offspring microbiota, and if so, what compartments of the offspring microbiota would be affected.

Hypothesis 2: Injected antibiotics administered in the last days of pregnancy alter the bacterial colonization of exposed offspring throughout the gastrointestinal (GI) tract. Tested compartments of the GI tract included the stomach, ileum, colon, and stool. See Figure 2 for the experimental protocol to address hypothesis 2.

We then focused on whether changes to the microbial colonization of the offspring were transient, or if they endured throughout the offspring's developmental period.

Hypothesis 3: Changes to the microbiota induced by parenteral antibiotics during late gestation will last throughout the offspring's developmental period. See Figure 3 for the experimental protocol to address hypothesis 3.

To determine whether the altered microbiota of offspring was the result of antibiotic exposure during gestation per se, or the result of being reared by a dam exposed to antimicrobials, offspring were exchanged shortly after delivery.

Hypothesis 4: Changes to the offspring microbiota occur as a result of altering the maternal microbiota, which does not recover during the offspring's developmental period. See Figure 4 for experimental protocol to address hypothesis 4.

Next, we considered if changes to the offspring microbiota affected their growth patterns as they aged.

Hypothesis 5: Changes in offspring microbiota during the developmental period as a result of maternal perinatal antibiotic exposure alter growth patterns and increase the likelihood of obesity and metabolic syndrome in adulthood when challenged by a Western-style diet. See Figure 5 for experimental protocol to address hypothesis 5.

Animal Husbandry

Housing

C57Bl/6 mice were obtained from Charles River Laboratories and bred in specific pathogen free conditions at the National Cancer Institute in Frederick, Maryland. All animal experimentation was approved by the Animal Care and Use Committee of the National Cancer Institute and the National Institutes of Health. Breeding pairs were transferred to the Bethesda campus of NIH to a specific pathogen free (SPF) environment. A SPF environment means that the mice are tested regularly for pathogens that are known to cause disease in mice that may interfere with experimental results. A list of the pathogens from which the mice in this experiment are certified free is included

in the Appendix 1. The mice were maintained in a temperature-controlled environment with 12-hour day and night cycles. Cages were changed weekly, and they were observed for signs of illness twice daily. The nutrient content of the diets used in this protocol, along with the manufacturers can be found in Appendix 2. In order to minimize the unequal introduction of microbes and the introduction of pathogenic microbes, all cages, water, and bedding were autoclaved before introduction to the mice. All foods were irradiated before feeding.

Co-fostering and Co-housing

In order to determine if the antibiotic exposure prior to delivery is causing the change in colonization or if it is the result of being reared by a mother who has been exposed to antibiotics, offspring were exchanged at three to five days of age so that each litter contained half of the dams own offspring and half fostered offspring from a mother who received the opposite treatment. Model, figure 4. As offspring that were being fostered were removed from their own dams and placed with another, they were at risk of rejection from the foster dam, thus possibly creating bias against the fostered offspring. In order to equalize this risk, and because the offspring must be identifiable for later testing, offspring that were not fostered, but left with their dam, were marked by cropping of the tail. At 3-5 days of life, offspring were weighed, and half of the offspring of each dam were placed with another dam in the opposite group that had a litter of the same approximate size and weight. The remaining pup's tails were cropped, and they were returned to their dams. Dams were encouraged to urinate on all of the offspring in order to encourage acceptance.

In order to determine the effect of cohousing on the long-term microbiota, the offspring were weaned to mixed cages of fostered and cropped no antibiotics and antibiotic-exposed. The offspring were all placed on the Western Diet protocol (figure 5) at weaning without further alterations to the protocol.

Mating, Gestation, and Weaning

Adult C57Bl/6-NCR female siblings were weighed and placed in individual cages with a single male for 5 days, at which time the male was removed. In 18 days, pregnant females administered 15mg/kg vancomycin, 4mg/kg gentamicin, and 125 mg/kg piperacillin-tazobactam intraperitoneally (IP) daily for 3 days, using a dosing weight of 20g (average weight of a female nulliparous mouse). Antibiotics were chosen for their broad-spectrum activity, because they were safe for both pregnant animals and fetuses, and because they are used for laboring women. Intraperitoneal injection was used because it gives more reliable systemic absorption than repeated intravenous injections in mice, which is more technically difficult. Mouse gestation last from 19-21 days. Daily antibiotic dosing was selected to simulate late gestation intravenous dosing that women receive as prophylaxis prior to delivery. Injections were stopped when the mouse delivered her offspring. Figures 1, 2-Models developed to test the impact of antibiotics on the maternal microbiota and on the offspring microbiota in different gastrointestinal compartments.

Stool was collected from dams and their offspring at weaning, which occurred at 3.5 weeks of age. Offspring were separated from their dams and placed in gender segregated cages with offspring that received the same treatment. They were then placed

on a Western Diet. The diet was created by Research Diets, Inc. (Product# D17033010) to mimic the diet of U.S. citizens as reported in the CDC, National Center for Health Statistics in 2010, containing 35% fat (10% saturated), 50% carbohydrates (15% fructose), 15% protein, and 16g fiber (Wright and Wang 2010). Diet ingredients available in Appendix 2. Weights and food consumption were monitored weekly.

Procedures and Sample Collection

All procedures took place in the laboratory of Dr. Giorgio Trinchieri, program director of the Cancer and Inflammation Program and head of the Cancer Immunobiology Section of the Center for Cancer Research at the Bethesda Campus of the National Institutes for Health.

DNA extraction

Stool was collected for microbiota analysis before weaning, after weaning, and again at 30 weeks of age when organs were harvested. Each mouse was placed in an individual container, weighed, and then stool was collected. Stomach, ileum, and colon contents were collected weekly for the first 6 weeks and at the 30-week harvest. All specimens were collected into 1.5ml Eppendorf tubes and snap frozen in dry ice. Tubes were stored at -80 degrees until analysis. DNA was extracted using the Mobio PowerMag Microbiome DNA/RNA isolation kit (cat#27500-4-EP) and the Eppendorf Epmotion liquid handler (Eppendorf, epMotion5075 and epMotion 5073).

16S rDNA library preparation and sequencing

The gene-specific sequences used in this protocol target the 16S V4 region (515F-806R). Illumina adapter overhang nucleotide sequences are added to the gene-specific sequence.

First PCR amplification (gene-specific amplification)

1) In sterile thin-walled tubes mix the following in a final volume of 25 ul.

Component	ul/rxn	Final conc.
Template DNA	10.5	100ng
Phusion High Fidelity PCR MM	12.5	1x
5uM Forward Primer	1	0.2uM
5uM Reverse Primer	1	0.2uM

2) Carry out 17 cycles of amplification using the following program;

98°C for 30 sec	1 cycle
98°C for 10 sec 60°C for 30 sec/1 degree decrease/cycle 72°C for 30 sec	10cycles
98°C for 30 sec 50°C for 30 sec 72°C for 30 sec	7
72°C for 7 min	1 cycle
Hold at 4°C	

1) PCR products were purified using Ampure XP beads (Beckman coulter Cat# A63881) at ratio 1:1. Final product were eluted with 50ul EB buffer (Qiagen Cat#19086)

Second PCR amplification (illumina Bar code amplification)

1) Mix the following in a sterile thin-walled tube in a final volume of 25ul

Component	ul/rxn	Final conc.
DNA (Purified PCR product of the 1st)	8.5	
2xPhusion High Fidelity	12.5	1x
2.5uM Index 1 (N7xx)	2	0.2uM
2.5uM index 2 (S5XX)	2	0.2uM
PCR-grade H2O	0	

2) Carry out amplification using the following program:

98°C for 30sec	1 cycle
98°C for 10 sec 55°C for 30 sec 72°C for 30 sec	8
72°C for 7 min	1 cycle
Hold at 4°C	

Purify the PCR products using Ampure XP beads with 1:1 ratio, elute with 25ul EB Buffer

3) Determine the concentration of library using KAPA Library quantification kit (cat#KK4873, Kapa Biosystems) on Quantstudio 6 Flex (Thermofisher).
4) Combine the same amount of samples in a sterile microcentrifuge tube. Quantify the pooled library with Qubit
5) The pooled library was sequenced on a Miseq machine with 10% phix spike in.

Metagenomic library preparation and sequencing

Nextera DNA Flex Library Prep reference guide, Document # 1000000025416 v04 from Illumina was followed with automated epMotion 5075.

Glucose and Insulin Tolerance Tests

Mice were placed in clean cages without food for 6 hours prior to testing. Baseline glucose and insulin measurements were obtained by taking 0.15 ml of blood via mandibular venous puncture. Mice were then administered 2g/kg of glucose in sterile water orogastric or 2units/kg of insulin in sterile water IP based on their weight on the day of the test. Blood was then sampled by nicking the tail at 15 minutes, 30 minutes, 60 minutes and 120 minutes after glucose or insulin administration. A Bayer Contour Next EZ Glucosometer was used to test glucose levels. After clotting, serum was separated and stored at -20 degrees until further analysis was performed. Glucose values were recorded and reported as both glucose curve and area under the curve. Differences were reported using two-way ANOVA or multiple t-tests with adjustments depending on the number of groups.

Harvest methods

After euthanasia with carbon dioxide per IACUC guidelines, blood was collected by cardiac puncture, and placed in serum separator tubes. The abdomen was then accessed in order to sever the inferior vena cava. The diaphragm was then opened and the ribs along the left side cut to reveal the heart, which was punctured in the left ventricle and injected with 20ml of phosphate buffered saline (PBS) in order to perfuse the liver. After perfusion, the liver was excised, and the largest lobe was placed in a histology a

cassette in 4% paraformaldehyde. Two small pieces of the next biggest liver lobe, and 2 ½ centimeter slices of the distal colon and the distal ileum were placed in RNAlater and stored at room temperature overnight before being frozen at -80 degrees. The intestine was then removed from the abdominal cavity and the fecal matter collected and frozen on dry ice before being stored at -80 degrees until analysis. Cecal contents were also collected in 20% glycerol and snap frozen and stored at -80 degrees.

RNA extraction for RNAseq

RNA was extracted from flash frozen liver tissue using the Qiagen RNeasy Plus Mini Kit, Catalog # 74136. Briefly tissue was lysed and homogenized in denaturing buffer then passed through a genomic DNA Eliminator spin column. After the addition of ethanol, the sample is then applied to an RNA binding column and then eluted with rnase-free water. The binding column isolates RNA longer than 200 nucleotides, thereby enriching for messenger RNA (mRNA). The mRNA was then quantified using nanodrop and assigned an RNA integrity number before proceeding to library preparation and sequencing.

RNA extraction for nanostring

Small pieces (~.5g) of GI tissues were cut from the sample, and homogenized in mirVana™ lysis buffer using the PowerLyzer 24 Homogenizer (Qiagen, Hilden, Germany, Cat. no. 13155) with ceramic 2.8 mm PowerBead tubes. RNA was extracted from lysate using the standard protocol from the mirVana Isolation Kit™ (ThermoFischer, Waltham, MA, Cat. no. AM1560). RNA was quantified with a Nanodrop instrument and equilibrated to 20ng/uL according to Nanostring manuals

(MAN-10056-02 CodeSet Hybridization Setup, MAN-10023-11 nCounter® XT Assay User Manual).

RNA library preparation and sequencing

RNA library preparation was accomplished using the Lexogen Quant Seq 3' mRNA-seq FWD library prep kit (Catalog #015.96) following manufacturer's instructions.

Histology

Liver tissue preserved in 4% paraformaldehyde for at least 24 hours, then rinsed and stored in PBS at 4 degrees. Two days before staining, the tissue was transferred to 10% sucrose solution overnight, then transferred again to 30% sucrose in PBS overnight before processing further. Slicing, Hematoxylin and Eosin staining, and cryopreservation were performed by Histoserv, Inc..

ELISA

Insulin was tested using 5 microliters of blood using the Low Range Ultra Sensitive Mouse Insulin ELISA Kit from Chrystal Chem High Performance Assays (Cat# 90080 Elk Grove Village, IL). Optical Densities measured using the Agelint Spetrophotometer. Optical Densities were interpolated from standards using a sigmoidal 4-point curve as per manufacturer's recommendation in GraphPad PRISM, version 8.00 for Macintosh, GraphPad Software, La Jolla California USA, www.graphpad.com.

Data Analysis and Bioinformatics

Weights

When comparing three or more groups, weights were analyzed using mixed modeling followed by Tukey's multiple comparisons test using GraphPad. Mixed

modeling was used rather than repeated measures ANOVA because of unequal group sizes. Multiple comparisons were made using simple effects at each time period. When comparing two groups statistical significance was determined at each time period using multiple t-tests with the Holm-Sidak method for correcting multiple comparisons, GraphPad Prism. An adjusted p-value of less than 0.05 was considered significant.

Glucose and Insulin Tolerance

When comparing three or more groups at each time period, 2way ANOVA was used with Tukey's multiple comparisons test. When comparing two groups at each time period, Mann-Whitney test was used. When comparing the glucose on insulin reaction over time, area under the curve values were compared using Wilcoxon for groups of two or Kruskal-Wallis for groups of three or more. All statistical tests were performed in GraphPad Prism with significance values of $p < 0.05$.

16s rDNA

The V4 region (515F-806R) of the gene encoding 16S rRNA was amplified using PCR and paired end (2x300bp) reads were generated on the Illumina MiSeq platform. The resulting data were analyzed using the DADA2 R package (Callahan et al. 2016) in order to generate sequence variants which were subsequently analyzed at the phylum, family, and genus levels. The results were visualized using a combination of the phyloseq (McMurdie and Holmes 2013), metagenomeSeq (Paulson et al. 2013), and JAMS (McCulloch, et al, unpublished) R packages. Differences between groups were assessed

using the Wilcoxon signed rank test (Wilcoxon 1945) and PERMANOVA (Anderson 2001). DESeq2 (Love et al. 2014) was used to find differentially abundant taxa.

The demultiplexed paired-end fastq files were pre-processed and analyzed using QIIME2 version 2.2017.8 (Caporaso et al. 2010). The DADA2 algorithm (Callahan et al. 2016), implemented in QIIME2, was used for error modelling and filtering the raw fastq files. Post denoising and chimera removal; a total of 3,855,460 sequences were retained for 20 samples with an average of 192,773 sequences per sample.

Taxonomic classification was performed using the QIIME2 feature-classifier plugin trained on the Silva 132 database (Quast et al. 2013). The Alpha and Beta-diversity analyses were performed using the diversity plugin at a sampling depth of 135,297.

Metagenomic

Sequencing reads were quality trimmed and adapter clipped using Trimmomatic (Bolger et al. 2014). Reads were then aligned to the *Mus musculus* genome (all chromosomes plus mitochondria) to filter out host reads with Bowtie2 (Langmead and Salzberg 2012). Unaligned reads were then assembled using Megahit version v1.1.3 (Li et al. 2015). The resulting contigs were annotated using Prokka v1.13 (Seemann 2014) also yielding the predicted proteome. The trimmed sequencing reads were aligned back to the metagenomic contigs using bowtie2 to gauge depth. Reads unaligned to the contigs (unassembled reads) were collected. The metagenomic contigs and the unassembled reads were taxonomically classified by k-mer analysis using kraken v1.0 (Wood and Salzberg 2014) with a custom-build database comprising of all the complete and draft genome sequences in GenBank of all Bacteria, Archaea, Fungi, Viruses, Protozoa plus

the genomes of *Mus musculus* and *Homo sapiens*. Each sequence classified (either contig or read) was attributed to its Last Known Taxon (LKT). The relative abundance of each Last Known Taxon in the sample was computed by the number of bases covering contigs belonging to that LKT plus the number of bases from unassembled reads belonging to that LKT divided by the total number of bases sequenced in that sample.

All statistical analyses were carried out in the R programming language (v3.5.0). Heatmaps were plotted using the ComplexHeatmap package v3.8 (Gu et al. 2016). The significance of differences in relative abundances between groups were calculated with the Mann-Whitney-Wilcoxon test and the p-values were adjusted by FDR.

RNAseq

Analysis of RNA was generated using Partek Flow® software. Briefly, unaligned reads were trimmed from the 3-prime end based on quality (Phred) score of 20 and minimum read length of 25. Trimmed reads were then aligned to the *Mus musculus* genome using the STAR aligner (Dobin et al. 2013). After post-alignment quality control, transcripts were quantified using Partek E/M transcript model mm10-RefSeq Transcripts 86-2018-08-01 with a minimum coverage threshold of 10. Feature counts less than 10 were excluded to reduce noise. Filtered transcripts were normalized using the addition of 1 to avoid zero values and the trimmed mean of M-values (TMM) method (Robinson and Oshlack 2010). Groups were compared by gender, by diet, and by antibiotic exposure. Partek GSA multi-modeling approach was used with differential analysis filter of p value ≤ 0.05; fold change < -2 or > 2. Clustered samples were reported using Hierarchical Clustering, Principal Component Analysis (PCA) (Jolliffe and Cadima 2016), t-

Distributed Stochastic Neighbor Embedding (t-SNE)(van der Maaten and Hinton 2008), and Pathway enrichment analysis using Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (Ogata et al. 1999).

Nanostring nSolver™

Transcript quantification and gene expression analysis were performed using the nCounter platform under the XT Assay protocol (see manual above). We used a custom multiplex Nanostring™ probe panel with 250-probes for genes of interests in intestinal epithelial regulatory pathways selected from KEGG pathway database, these included tight junction, cytoskeletal morphology, and colorectal carcinoma, and others (see Supplementary Materials – Probe Panel Annotations). Design parameters of the human orthology version of this probe panel was described previously (Robinson and Henderson 2018), with cell-culture results from that probe panel described in (Robinson et al. 2018). Nanostring nCounter® system is a semi-automated system which uses multiplexed, fluorescent barcode-labelled probe hybridization, using an optical scanning and read counting method. Input RNA of 100ng was used as recommended, the method does not require amplification of input RNA. Analysis of read-count data was performed using the Nanostring nCounter nSolver™ 4.0 (Nanostring MAN-C0019-08) with Nanostring Advanced Analysis Module 2.0 plugin (Nanostring MAN-10030-03), following the Nanostring Gene Expression Data Analysis Guidelines (Nanostring MAN-C0011-04). Advanced Analysis Module Software uses open-source R packages for QC, Normalization, Differential Expression analysis, and Gene Set Enrichment analysis. Samples failing QC were excluded from the analysis. Normalization was performed

using the geNorm algorithm, with selection of the 10 genes with least variance in expression between samples and treatments. All raw and normalized data are pending submission to the NCBI Gene Expression Omnibus database. All QC, normalization, differential expression, and pathway analysis data and graphical outputs are available upon request. Differential expression utilizes a data modeling algorithm that preferentially applies the optimal method per gene 1) Mixture negative binomial model, 2) Simplified negative binomial model, 3) Log-linear model, in that order, for determination of DE. FDR p-value adjustment was performed with Benjamini-Yekutieli method. Under the data model, predictor co-variables included: Tissue type (Colon, Cecum, Illeum, Jejunum, Duodenum, Stomach), Diet (Standard, Western), Antibiotic Treatment (Control, Oral, Intraperitoneal).

Strengths and Limitations

Mice were chosen for this experiment because genetically controlled strains are available, and they have a short gestation and large litter size that allows frequent repetition in similar experimental conditions. Although mouse and human placentation is structurally different throughout the first 2/3 of the pregnancy, near the end of gestation, they are remarkably similar. They differ in that maternal and fetal placental capillaries are in parallel in mice allowing for more efficient exchange between maternal and fetal circulations, though placentation is shallower; while humans have deeper placental invasion, but a less efficient villous structure for exchange (Malassiné et al. 2003; Clark 2014). Endocrine functions in mouse and human gestation are quite different as are important immune cell subsets (Malassiné et al. 2003). For the purposes of this work however, a hemotrophic placenta is sufficient to deduce the effects of injected antibiotics

on both mother and infant, especially as the focus is on bacterial colonization. Bacteria, being niche specialists, often co-evolve with their hosts in order to take advantage of unique opportunities present as a result of feeding, environment, and hygiene practices (Miller et al. 2007). Bacterial species can be expected therefore to differ between mice and humans, but changes to the normal pattern may be observed and projected to emulate changes to the microbial environment of similar species. As with any animal model, validation in humans must occur before suppositions are made about findings that apply to both species.

The availability of specific pathogen free environment reduces the likelihood of pathogenic bacteria in the environment contributing to phenotypic phenomena thereby confounding variables of interest to the study. The pathogen-free environment is artificial, however, and may make conclusions less relevant to organisms that are not so insulated from normal disease and competition for resources. The advantage of limiting possible confounders is to gain insight into the mechanism of action leading to phenotypic observations. In applying insights gained, however, one must be aware that each confounder may contribute alterations that affect the outcomes being studied.

One challenge to this study was the difficulty, as in pregnant women, of predicting the exact time of birth. Mouse gestation is 19-21 days long. In order to be sure that the mice were exposed to the antibiotics before delivery, antibiotics were started at 18 days, and administered once per day until delivery. This necessarily meant that some mice would receive more doses of antibiotics than others. Variable antibiotic exposure also occurs in pregnant women, where the goal is to receive at least 4 hours of antibiotics for GBS prophylaxis prior to delivery, but often occurs over many hours or even days.

Another challenge was that although mice demonstrated differences in insulin levels to maintain normal fasting glucose, the mice did not differ in growth on a regular chow diet, which is based on the minimum requirements needed to maintain healthy mice. A diet that emulated the macronutrients that western populations, such as the population of the United States was therefore created to better understand the interaction between altered microbial colonization and growth in the circumstances of less than ideal nutrition. The western diet was successful at inducing obesity in mice, though not equally between genders. Males became immediately obese and glucose intolerant, though not different by antibiotic exposure. Male mice did, however, still have elevated insulin levels if exposed to antibiotics during gestation. Females were somewhat resistant to the western diet, and some females in both antibiotic-exposed and control groups remained slim. Although it is also true that in humans a poor diet does not always predict obesity, the gender differences observed in mice do not predict the human experience. It is difficult to know whether the susceptibility to obesity upon exposure to the western diet was so extreme that differences that might exist as a result of dysbiosis in males were masked. In females, it is also not straightforward, as not all mice responded with altered growth patterns. Though the mice were genetically identical, and many confounding variables were controlled, more research will be required to understand the mechanism by which the microbiota influences the growth of both male and female offspring of antibiotic treated mothers.

The greatest strength of this study was the environment in which it was conducted and the personnel that were available for consultation and assistance. The Trinchieri lab has a microbiome core facility within it where DNA extraction and sequencing are

readily accessible. There are also scientists, statisticians, and bioinformaticists available both in the core and in the lab for help and consultation. The resources available at NCI, NINR, and in the NIH community are unparalleled.

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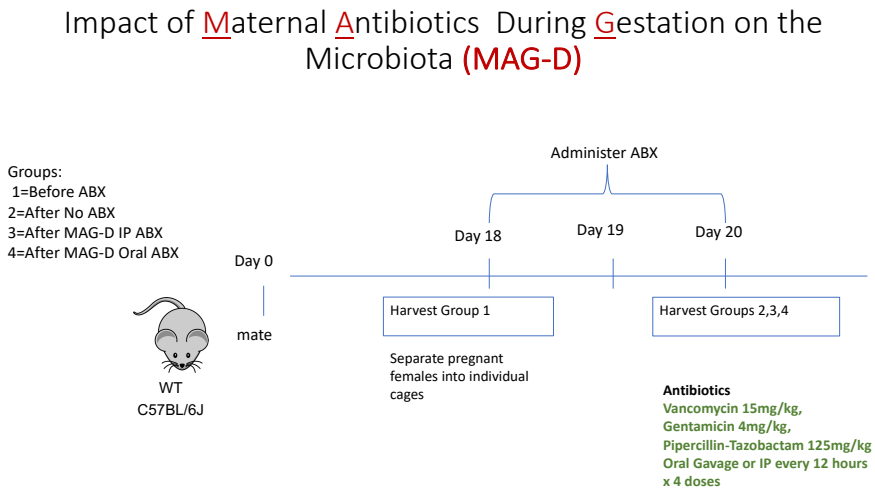

Figure 1. Experimental protocol to address the impact of intraperitoneal antibiotics administered to pregnant female mice during the last days of gestation on maternal microbiota.

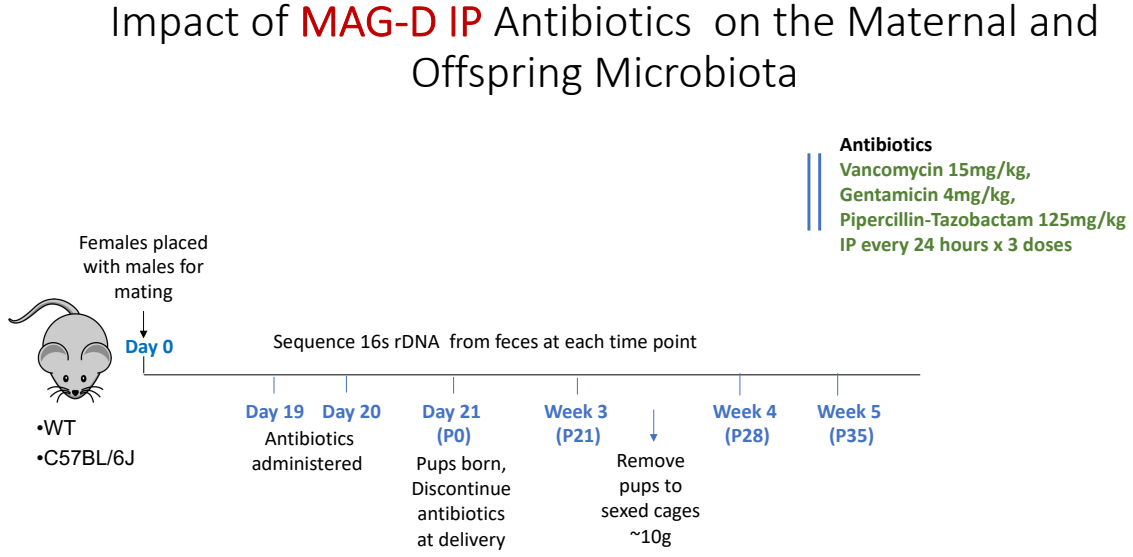

Figure 2. Experimental protocol to determine the impact of intraperitoneal antibiotics administered to pregnant female mice during the last days of gestation on maternal and offspring microbiota throughout the offspring developmental period.

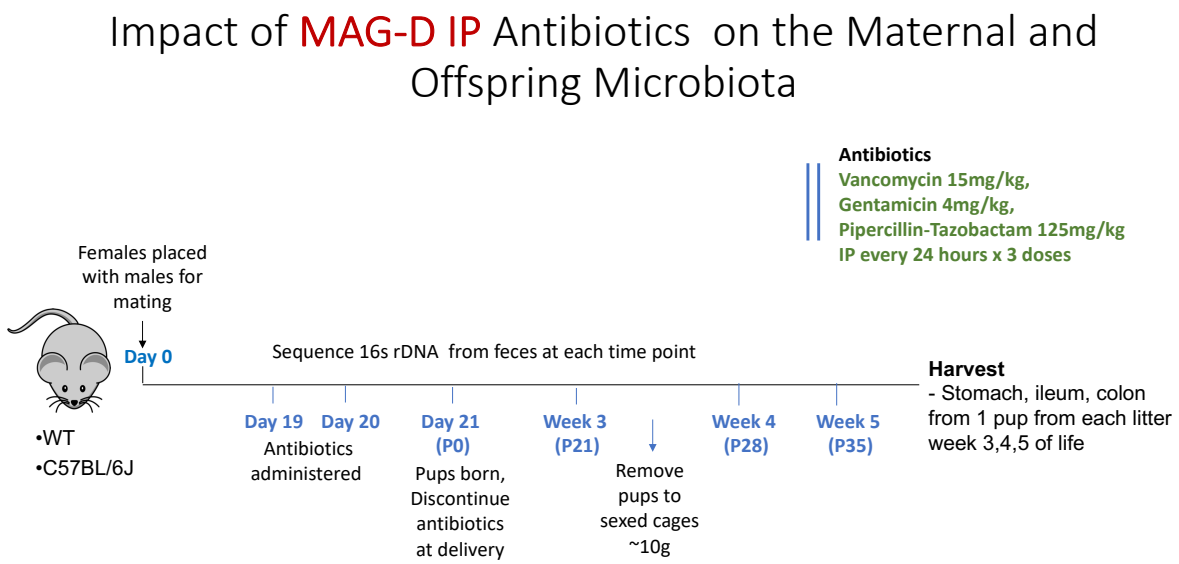

Figure 3. Experimental protocol to determine the impact of intraperitoneal antibiotics administered to pregnant female mice during the last days of gestation on maternal and offspring microbiota throughout the offspring developmental period.

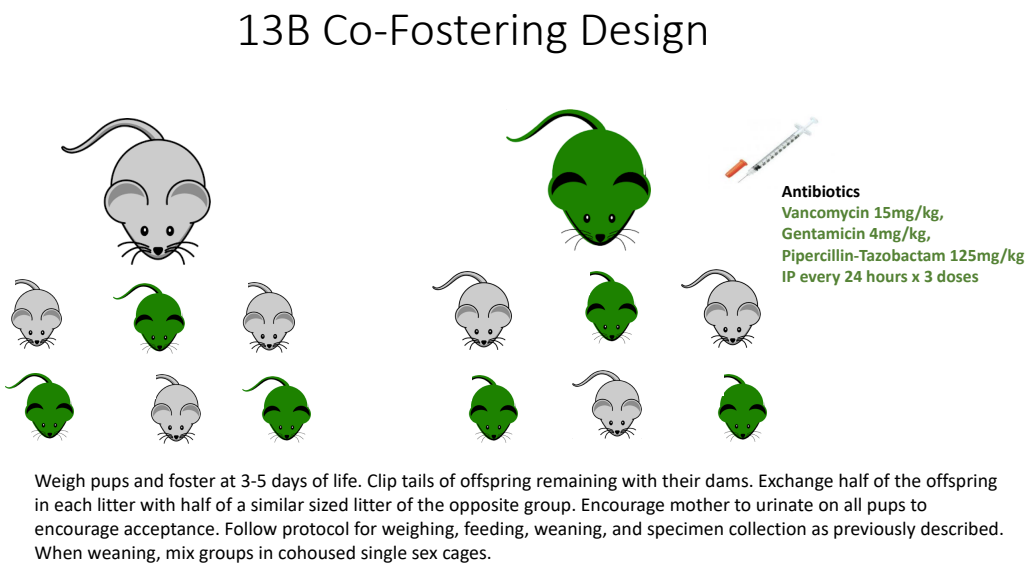

Figure 4. Experimental protocol to determine if changes to the offspring microbiota occur as a result of altering the maternal microbiota or as a result of exposure to antibiotics during gestation.

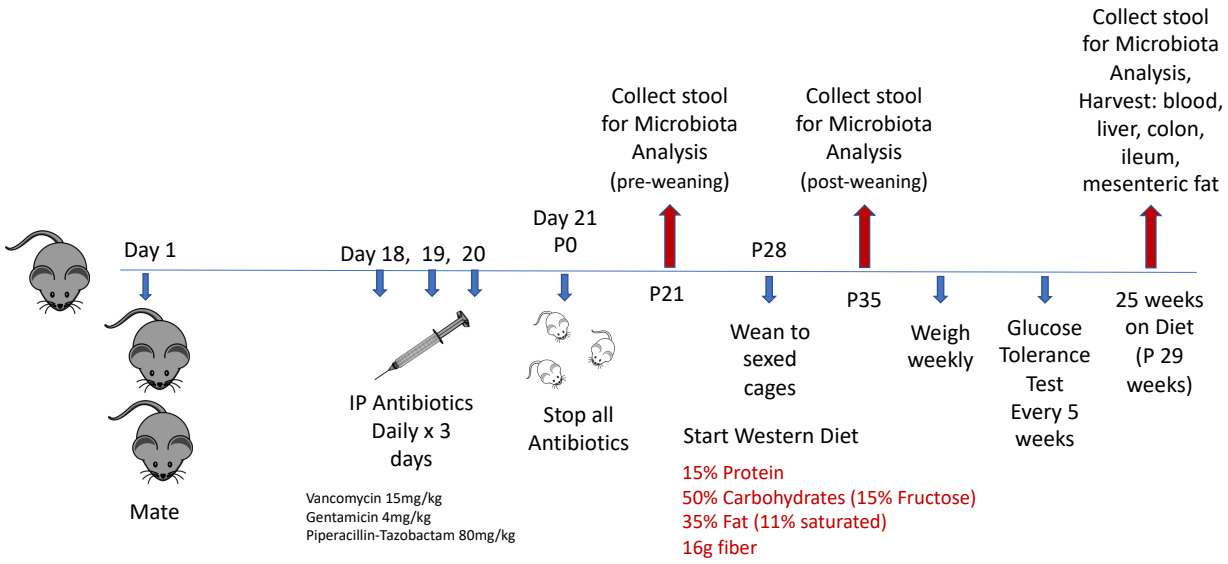

Figure 5. Experimental protocol to determine if changes in offspring microbiota during the developmental period as a result of maternal perinatal antibiotic exposure alter growth patterns and increase the likelihood of obesity and metabolic syndrome in adulthood when challenged by a Western-style diet.

Appendix 1 NCI Rodent Excluded Agents – 2017

Viral:

Mouse

sendai virus (Send)
murine hepatitis virus (MHV)*
rotavirus/epizootic diarrhea of infant mice virus (EDIM)*
lymphocytic choriomeningitis virus (LCMV)
mouse encephalomyelitis virus (TMEV/GDVIII)*
reovirus- type 3 virus (Reo-3)*
minute virus of mice (MVM)*
mouse parvovirus (MPV)*
pneumonia virus of mice (PVM)*
mouse adenovirus (MAD)*
mouse cytomegalovirus (MCMV)
mouse thymic virus (MTV)
mouse pneumonitis virus (K)
polyoma virus (Poly)
hantaan virus/all hanta viruses (HAN)
ectromelia virus (Ectro)
Lactate Dehydrogenase Elevating Virus (LDEV)

Rat

sendai virus
reovirus 3
sialodacryoadenitis virus (SDAV)
rat corona virus (RCV)
Kilham's rat virus (KRV)
Toolan's H-1 parvovirus (H-1)
rat parvovirus (RPV)
Rat minute Virus (RMV)

Ectoparasites*:

All fur - Including, but not limited to:

Mycoptes musculinus
Radfordia ensifera
Myobia musculi
Myobia ratti
1 Trichoecius romboutsi
Notoedres muris
Psorergates simplex

All blood sucking mites – Including, but not limited to:

Ornithonyssus bacoti

All lice- Including, but not limited to:

Polyplax serrata
2 Polyplas spinulosa
Hoplopleura pacifica

Endoparasites*:

Giardia spp.
Spironucleus spp.
All nematodes- Including, but not limited to:
Aspiculuris tetraptera
Syphacia obvelata
Syphacia muris
All cestodes- Including, but not limited to:
Hymenolepis nana
Hymenolepis diminuta
Bacterial:
Salmonella spp.
Citrobacter rodentium (formerly know as *Citrobacter freundii* strain 4280)
3 Streptobacillus moniliformis
Clostridium piliforme
Streptococcus pnumoniae
4 Corynebacterium kutscheri
Cilia-associated respiratory bacillus (CARB)
Helicobacter species (except ACRF)*
Other:
Mycoplasma pulmonis
Mycoplasma arthriditis
Encephalitozoon cuniculi

Appendix 2

Chow Diet

7013

ENVIGO

NIH-31 Modified Open Formula Mouse/Rat Sterilizable Diet

Product Description- 7013 is a fixed formula, autoclavable diet manufactured with high quality ingredients and designed to support growth and reproduction of rodents. 7013 is supplemented with additional vitamins to ensure nutritional adequacy after autoclaving. **Also available irradiated (7913).**

Ingredients (in descending order of inclusion)- Ground wheat, ground corn, ground oats, wheat middlings, fish meal, dehulled soybean meal, soybean oil, dehydrated alfalfa meal, corn gluten meal, dicalcium phosphate, brewers dried yeast, calcium carbonate, iodized salt, choline chloride, magnesium oxide, kaolin, ferrous sulfate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, thiamin mononitrate, niacin, calcium pantothenate, vitamin E acetate, vitamin A acetate, copper sulfate, zinc oxide, riboflavin, pyridoxine hydrochloride, vitamin B_{12} supplement, vitamin D_3 supplement, calcium iodate, biotin, folic acid, cobalt carbonate.

Standard Product Form: Pellet

Macronutrients		
Crude Protein	%	18.0
Fat (ether extract) [a]	%	6.2
Carbohydrate (available) [b]	%	45.0
Crude Fiber	%	4.0
Neutral Detergent Fiber [c]	%	13.6
Ash	%	6.2
Energy Density [d]	kcal/g (kJ/g)	3.1 (13.0)
Calories from Protein	%	23
Calories from Fat	%	18
Calories from Carbohydrate	%	59
Minerals		
Calcium	%	1.1
Phosphorus	%	1.0
Non-Phytate Phosphorus	%	0.7
Sodium	%	0.3
Potassium	%	0.6
Chloride	%	0.5
Magnesium	%	0.2
Zinc	mg/kg	47
Manganese	mg/kg	155
Copper	mg/kg	13
Iodine	mg/kg	2
Iron	mg/kg	270
Selenium	mg/kg	0.30
Amino Acids		
Aspartic Acid	%	1.5
Glutamic Acid	%	3.2
Alanine	%	1.1
Glycine	%	1.0
Threonine	%	0.7
Proline	%	1.5
Serine	%	0.9
Leucine	%	1.4
Isoleucine	%	0.8
Valine	%	0.8
Phenylalanine	%	0.8
Tyrosine	%	0.7
Methionine	%	0.4
Cystine	%	0.3
Lysine	%	0.8
Histidine	%	0.4
Arginine	%	1.0
Tryptophan	%	0.2

Vitamins		
Vitamin A [e, f]	IU/g	24.2
Vitamin D_3 [e, g]	IU/g	4.2
Vitamin E	IU/kg	45
Vitamin K_3 (menadione)	mg/kg	22
Vitamin B_1 (thiamin)	mg/kg	76
Vitamin B_2 (riboflavin)	mg/kg	7
Niacin (nicotinic acid)	mg/kg	87
Vitamin B_6 (pyridoxine)	mg/kg	9
Pantothenic Acid	mg/kg	39
Vitamin B_{12} (cyanocobalamin)	mg/kg	0.06
Biotin	mg/kg	0.30
Folate	mg/kg	2
Choline	mg/kg	1890
Fatty Acids		
C16:0 Palmitic	%	0.9
C18:0 Stearic	%	0.2
C18:1ω9 Oleic	%	1.3
C18:2ω6 Linoleic	%	2.5
C18:3ω3 Linolenic	%	0.3
Total Saturated	%	1.2
Total Monounsaturated	%	1.5
Total Polyunsaturated	%	2.8
Other		
Cholesterol	mg/kg	50

[a] Ether extract is used to measure fat in pelleted diets, while an acid hydrolysis method is required to recover fat in extruded diets. Compared to ether extract, the fat value for acid hydrolysis will be approximately 1% point higher.

[b] Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates.

[c] Neutral detergent fiber is an estimate of insoluble fiber, including cellulose, hemicellulose, and lignin. Crude fiber methodology underestimates total fiber.

[d] Energy density is a calculated estimate of *metabolizable energy* based on the Atwater factors assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrate.

[e] Indicates added amount but does not account for contribution from other ingredients.

[f] 1 IU vitamin A = 0.3 µg retinol

[g] 1 IU vitamin D = 25 ng cholecalciferol

For nutrients not listed, insufficient data is available to quantify.

Nutrient data represent the best information available, calculated from published values and direct analytical testing of raw materials and finished product. Nutrient values may vary due to the natural variations in the ingredients, analysis, and effects of processing.

Teklad Diets are designed and manufactured for research purposes only.

0915

Teklad Diets + Madison WI + envigo.com + tekladinfo@envigo.com + (800) 483-5523

Appendix 2 Western Diet

D17033009-10

Formulated by:
Research Diets, Inc.
3/30/2017

Rodent Diets with 15 kcal% Protein and 10 kcal%
or 35 kcal% Fat with 15 kcal% Fructose and 16g Cellulose/4058kcal

Product #	**D17033009**		**D17033010**	
%	gm	*kcal*	gm	*kcal*
Protein	*14*	*15*	*17*	*15*
Carbohydrate	72	75	58	50
Fat	*4*	*10*	*18*	*35*
Total		*100*		*100*
kcal/gm	3.8		4.6	
Ingredient	**gm**	***kcal***	**gm**	***kcal***
Casein	149.7	*599*	149.7	*599*
L-Cystine	2.3	*9*	2.3	*9*
Corn Starch	551	*2204*	294	*1176*
Maltodextrin 10	200	*800*	50	*200*
Sucrose	0	*0*	0	*0*
Fructose	0	*0*	153	*612*
Cellulose	50	*0*	16	*0*
Soybean Oil	45	*405*	10	*90*
Lard	0	*0*	0	*0*
Butter, Anhydrous	0	*0*	148	*1332*
Mineral Mix S10026	10	*0*	10	*0*
DiCalcium Phosphate	13	*0*	13	*0*
Calcium Carbonate	5.5	*0*	5.5	*0*
Potassium Citrate, 1 H2O	16.5	*0*	16.5	*0*
Vitamin Mix V10001	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*
FD&C Yellow Dye #5	0.05	*0*	0	*0*
FD&C Red Dye #40	0	*0*	0.05	*0*
FD&C Blue Dye #1	0	*0*	0	*0*
Total	**1055.05**	***4057***	**880.05**	***4058***

Research Diets, Inc.
20 Jules Lane
New Brunswick, NJ 08901 USA
info@researchdiets.com

PrescottS01.for.xls

Intrapartum Prophylactic Antibiotics have Lasting Impact on Offspring Microbiota

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Keywords: antibiotics, intrapartum, microbiota, pregnancy, offspring. dysbiosis, gestation

Abstract

Nearly half of all pregnant women receive prophylactic antibiotics prior to delivery for the prevention of neonatal early onset sepsis and maternal surgical site infection. Studies have shown that oral antibiotics administered to infants alter the microbiota resulting in increased obesity, allergy, asthma, and neurobehavioral problems. Little research has focused, however, on the impact of the prophylactic antibiotics given to mothers prior to delivery, which are typically parenteral and of limited duration. Here we use a murine model to investigate the effects of antepartum antibiotic administration on the microbiota of C57Bl/6 pregnant dams and their offspring. After receiving 1-3 doses of antibiotics in the last days of gestation, dams had markedly reduced fecal alpha diversity with a notable reduction of species in the *Bacteroidetes* phylum, while species in the *Firmicutes* phylum increased. Offspring of exposed mice had reduced bacterial diversity and altered colonization in tested gastrointestinal compartments into maturity. It has been shown that disruptions to the microbiota during the early developmental period are more likely to initiate long-term changes in metabolism and immunity. Interestingly, when fostered shortly after birth, it was the mother's antibiotic exposure that that shaped the microbiota of fostered offspring, rather than direct exposure to antibiotics in utero. The ability to influence the microbiota by postnatal exposure may play an important role in ensuring that infants have the ability to colonize with the full spectrum of microbes needed for long-term healthy metabolic and immune function.

Introduction

The human microbiota is the community of organisms that live in and on the human body and interacts with the host to influence metabolism and immunity (Sanz and Moya-Pérez 2014; Burcelin 2016). These organisms evolve within the host throughout the lifespan impacting growth and immune regulation. The microbiota is established first from the mother during gestation and develops over the first few years into the adult human pattern. Infants whose colonization is disrupted by antibiotic exposure have altered microbial composition that may persist for years (Noel T Mueller et al. 2015). These years of life represent critical developmental periods that may change growth patterns and immune function across the lifespan. Prophylactic antibiotics are administered to the 1.2 million women receiving cesarean sections, and to the 975,000 pregnant women who are colonized with *Group beta streptococcus* (GBS) in the United States each year (Hamilton et al. 2015). Febrile women and those with prolonged rupture of membranes, or other risk factors are also treated prior to delivery (Committee on Practice Bulletins-Obstetrics 2018). While studies indicate that oral antibiotics administered to the infant have lasting effects on microbial colonization and are associated with obesity and asthma (N T Mueller et al. 2015; Russell et al. 2012), the impact of prophylactic antibiotics parenterally administered to the mother on the maternal and infant microbiome has not been well studied. It is important to understand the changes that occur in the developing newborn because of antimicrobial prophylaxis to the mother, as these changes occur at a critical period when the newborn is first being colonized, and these changes may persist or influence the future colonization of the developing infant. Additionally, an understanding of how various drugs and routes of

administration affect the resident microbes in specific compartments of the gastrointestinal tract may lead to therapeutic interventions to the mother or infant which promote more targeted antimicrobial therapy or encourage re-colonization with specific microbes that are advantageous to growth and immune programming.

Results

Limited exposure to both oral and parenteral antibiotics alters fecal and colonic microbiota composition in pregnant mice

We studied the fecal and colonic microbiota of pregnant mice in a specific pathogen free (SPF) environment during the last 3 days of gestation. C57Bl/6 female litter mates were obtained from the Jackson Laboratory at 5-6 weeks of age and allowed to acclimate to the National Institutes of Health (NIH) Bethesda campus mouse facility for 3 weeks. Females were then separated to individual cages at 9 weeks and placed with males for 5 days to induce pregnancy, after which the males were removed. Each female remained isolated throughout the remainder of her gestation. Antibiotics were initiated on the 18th day of gestation. Vancomycin 15mg/kg, gentamicin 4mg/kg, and piperacillin-tazobactam 125mg/kg were administered every12 hours by oral gavage or intraperitoneal (IP) injection. The fecal microbiota was sampled before antibiotic administration, and on day 20 of gestation, just prior to expected delivery. After euthanasia, the microbiota of the vagina, colon contents, and colonic mucosa were analyzed. Fecal profiling from the colon contents revealed reduced bacterial diversity (Fig. 1A) and large microbiota composition differences between pregnant controls and those receiving oral and IP antibiotics (Fig. 1B and C). Antibiotic-treated pregnant dams had significantly reduced

relative abundance of fecal *Bacteroidetes* and expanded *Firmicutes* populations at the time of delivery (Fig. 1D). Phyla level changes were driven by decreases in the relative abundance of the genera *Lachnospiracaea*, *Alistipes*, and *Bifidobacterium*, and increases in the genera *Lactobacillus*, *Faecalibaculum*, and *Enterococcus* in treated dams (Fig. 1E). There was low bacterial abundance in all vaginal samples, but increased diversity driven by increased *Proteobacteria* in dams exposed to IP antibiotics (Fig. 2A-B). Changes in vaginal bacterial populations did not create distinct clustering when examined by principal coordinate analysis (C).

Microbes were differentially affected by antibiotics and route of administration depending not only on location within the gastrointestinal tract, but also depending on the tissue layer examined. Antibiotic-treated mice were dominated by Firmicutes, but genera within the phylum were differentially impacted by antibiotic therapy and route of administration. Significant differences between antibiotic treated dams and untreated controls increased from fecal samples to colon content samples to samples form the colonic mucosa (Figs. 3A-C). Most fecal bacterial populations were reduced in the presence of antibiotics, but the facultative anaerobes, *Lactobacillus* and *Staphylococcus,* increased in abundance in feces (Fig 3E-F). Enterococcus is increased in colon contents and in the mucosa (Figs. 3G). Ileal niche specialists were most disturbed by antibiotic administration (Fig 4A-D). Many bacteria such as *Lachnoclostridium*, *Ruminoclostridium*, and *Akkermansia* were reduced to nearly zero, while some, such as *Bifidobacteria* were affected differentially depending on route of administration (Fig. 4E-G). *Bifidobacteria* were eliminated in the mucosa of mice receiving oral antibiotics, while IP antibiotics reduced the ileal content population (Fig 4 H-I). The only population to

increase in the presence of antibiotics was the *Gammaproteobacteria*, *Acinetobacter* (Fig. 4J).

Offspring exposed to antenatal antibiotics have decreased alpha and increased beta diversity throughout the gastrointestinal tract during development

To determine whether limited exposure to perinatal antibiotics altered the microbiota of offspring, oral antibiotics, vancomycin, neomycin, and doripenem were administered in water starting on day 14 of gestation. Alternatively, vancomycin, gentamicin, and piperacillin-tazobactam were administered daily IP for a maximum of 3 doses as described above between day 18 and 20 of gestation. Antibiotics were discontinued at delivery. After delivery, maternal feces were sequenced weekly until the offspring were weaned. Dams had reduced alpha diversity as measured by observed operational taxonomic units (OTUs) throughout the offspring's developmental period (Fig 5A). Offspring were weaned to sexed cages at 3.5 weeks of age. Dams that were not exposed to antibiotics maintained a stable microbial colonization throughout the offspring developmental period, while those exposed to antenatal antibiotics had large differences in colonization that did not resolve within the 6-week testing period (Fig 5B). Offspring stomach contents, ileal contents, colon contents, and feces were sequenced using 16s rDNA before and after weaning. Offspring had increased beta diversity in all tested gastrointestinal compartments for up to 6 weeks after delivery if exposed to antibiotics before delivery (Fig. 6A-C). Offspring exposed to antenatal antibiotics had reduced alpha diversity that did not resolve before maturity (Fig 6D). Unsupervised clustering of fecal bacterial populations in offspring exposed to antenatal antibiotics reveals the extent of

environmental remodeling imposed by oral or injected antibiotic administration prior to delivery (Fig 7A-B).

To determine if offspring bacterial populations were altered in response to more frequently prescribed, but less broad-spectrum, prophylactic antibiotics, the protocol was repeated using ampicillin 100mg/kg and cephazolin 60mg/kg IP on days 18-20 of gestation. Offspring had similar alpha diversity to control offspring (Fig. 8A), but increased beta diversity as measured by unweighted Unifrac at weaning (Fig. 8B) and at 5 weeks of age (Fig. 8C).

Foster mother/environment determines offspring microbiota composition

We exchanged half of the offspring from dams exposed to IP antenatal antibiotics with half of the offspring from dams without antibiotic exposure to determine if prenatal antibiotic exposure or postnatal rearing environment drove the colonization of offspring (Fig. 9). The birth mother's antibiotic exposure status had no effect on fostered offspring colonization (Fig. 10A). The microbiota of fostered offspring resembled that of the foster dam and cohoused siblings (Fig. 10B). Offspring reared by control dams had higher *Bacteroidetes* and lower *Firmicutes* at weaning than their natural siblings reared with antibiotic-treated mothers (Fig. 11). Pups were fostered at 3-5 days of life. At 7-10 days of life, dams were separated from offspring for 4 hours, then given oxytocin. Breastmilk was extracted to determine drug levels. Breastmilk had no residual antibiotics detectable at 7-10 days of life by mass spectrometry (data not shown).

Repeated pregnancies with antibiotic exposure led to increasing dysbiosis in offspring

To investigate the effects of repeated pregnancies with prophylactic antibiotic exposures, we treated female dams with oral antibiotics during the last week of pregnancy as described above in two consecutive pregnancies. Offspring were compared to offspring of controls that received no antibiotics during gestation, and offspring whose dams received antibiotics during their first gestation. Mice reared by dams with two exposures clustered uniquely from those reared by dams with one exposure and those without antibiotic exposure (Fig12A). Bacteria that were differentially abundant as a result of antibiotic exposure were increased in offspring reared by dams of with more than one exposure (Fig. 12B).

Offspring exposed to antibiotics during gestation had reduced fecal alpha diversity and increased fecal beta diversity up to 29 weeks of age

Offspring of dams that received antenatal antibiotics as well as those that did not were weaned to sex specific cages at 3.5 weeks of age. They were placed on a regular chow or western diet to evaluate the long-term effects on microbial colonization that result from antibiotic-driven changes during development. The western diet was used to examine the microenvironmental response to dietary challenges in the setting of altered microbial colony formation. At 29 weeks of age, offspring continued to have decreased alpha diversity if exposed to IP or oral antibiotics during the last days of their gestation (Fig.13A). Diet was responsible for approximately 20% of the variance in beta diversity,

but antenatal antibiotic exposure continued to account for approximately 10% of the variance in beta diversity 29 weeks after birth (Fig 13B).

Discussion

Changes in the microbiota of humans and animals have mostly been studied using oral antibiotics because this is the most common route of administration in humans, and because of the ease of oral administration in animals. The current study confirms that antibiotics administered IP, with phamacokinetics similar to intravenous administration, do affect the maternal microbiota similar to orally administered antibiotics. Additionally, the short duration of antibiotic exposure did not prevent the antibiotics from reducing bacterial diversity and altering bacterial population dynamics in the maternal gastrointestinal tract. After only 3 days of treatment, both oral and IP antibiotics reduced maternal fecal *Bacteroidetes* while expanding the *Firmicutes* populations. Changes in population dynamics were transferred to offspring by the mother and resulted in early dysbiosis that remained dysbiotic for at least 30 weeks. Vaginal bacterial populations had fewer significant alterations but showed a trend toward increased *Proteobacteria*. As reductions in fecal *Bacteroidetes* and increases in *Proteobacteria* are also seen in human children after cesarean section and exposure to maternal intrapartum prophylaxis (Jakobsson et al. 2014), antibiotic exposure may represent the driver of bacterial population changes more than lack of exposure to vaginal species. Increases in the *Firmicutes/Bacteroidetes* ratio has been correlated with childhood and adult onset obesity (Koliada et al. 2017; Riva et al. 2016), while decreases are associated with autoimmune disorders such as systemic lupus erythematosus (Hevia et al. 2014). The mechanisms by

which the intestinal bacteria alter host growth patterns and immune responses has yet to be fully elucidated, but brief exposure to intravenous antibiotics used as prophylaxis has long been thought to have minimal negative repercussions on the mother or her infant. Identifying the ways in which bacteria colonize the newborn and establish a healthy ecology may be vital to long-term human health and needs more intensive mechanism-based research so that therapies may be devised to assist newborns whose mothers require antibiotic treatment to recolonize appropriately to prevent maternal or neonatal morbidity.

This study revealed that changes to the microbiota differ depending on the route of antibiotic administration and on the sample type examined. Although the microbiota of excretions is often the only microbiota available for study in humans, bacteria that reside in mucosa, or adhere to tissue may be as important for interacting with host metabolism and immunity. Deranged microenvironments in different body sites may promote unique consequences depending on the location and function of microbes that normally occupy that niche. *Proteobacteria*, a driver of inflammation (Rizzatti et al. 2017), was elevated in the vagina, colon, and ileum, and ileal mucosa of pregnant mice exposed to antibiotics. In the case of antibiotic exposure of short duration, adherent bacteria may be more or less susceptible to antibiotics depending on the pharmacokinetics of the drug and the accessibility of the drug to bacteria residing in physically less accessible locations such as within or beneath the mucosa. Adherent bacteria are adapted to maintain their position in the gastrointestinal tract without succumbing to the forces of movement natural to their environments, and do not compete for nutrients with luminal species (Derrien et al. 2011). They therefore often have more direct interface with the host. Luminal bacteria

influence growth and immunity through the fermentation of otherwise inaccessible nutrients and the production of immunomodulatory metabolites, which are taken up during digestion (Brestoff and Artis 2013). Feces only revealed a small portion of the changes resulting from antibiotic treatment. Both luminal and mucosal microbiota were disturbed in both mothers and offspring after antibiotic exposure. As most human studies are only able to study feces, more animal studies are needed to understand the effect of maternal antimicrobial prophylaxis on colonizing microbes. Understanding which bacteria are affected by individual antibiotics and the ecology of each niche may provide insight into therapeutics that specifically target pathogenic bacteria while sparing bacteria that provide nutrients, helpful metabolites, or additional barriers against pathogenic species.

Culture-based methods have been the gold standard for diagnosis of bacterial infection. Current therapy for suspected bacterial infection involves treating with broad spectrum antibiotics until culture results are available and medications can be adjusted. Often, however, broad spectrum antibiotics are prescribed on the basis of symptoms or for prophylaxis. Exposure to broad-spectrum antibiotics may alter the microbial ecology at the mucosal or tissue level thereby increasing the risk of infiltration with inflammatory microbes and microbial products. Targeted therapy based on molecular methods of rapid detection may be preferable to short duration broad spectrum solutions.

Bacteria whose relative abundance increased after maternal antibiotic exposure included *Lactobacillus*, *Enterococcus*, and *Staphylococcus*. All three genera are notable for biofilm formation (Paharik and Horswill 2016; Salas-Jara et al. 2016; Holmberg and Rasmussen 2016). Biofilm formation by commensal bacteria, such as *Lactobacillus*

reuteri, are thought be selected by epithelial cells in rodents to help to maintain stable ecosystems by outcompeting or interfering with would-be pathogens (Frese et al. 2013). Biofilms are more frequently associated with bacterial pathogenesis in humans, however, as the human colon has a thick and fast-growing mucous layer that challenges biofilm formation (Ahn and Prince 2017; de Vos 2015). Nevertheless, biofilms also serve a protective role for bacteria, increasing antimicrobial tolerance by providing a complex structure that shields surface-attached communities from antimicrobials and host immune responses; and providing a pool of antimicrobial resistance genes in close proximity from a collection of communicating heterogenous cells (Mah 2012). Thus, antibiotic resistance is a prominent characteristic of biofilm-forming bacteria. The increased relative abundance of biofilm formers in antibiotic-treated dams seen in this study may therefore be a result of increased tolerance or resistance to the antibiotics. Short duration broad-spectrum antibiotics may represent an increased risk for selecting antibiotic resistant bacteria for colonization of newborns with prenatal antibiotic exposure. As many lactobacillus strains show high level of resistance to vancomycin and cephalosporins, and these antibiotics are often chosen for prophylaxis or broad-spectrum treatment of suspected infection, we may be exerting selective pressure for antibiotic resistant organisms as early colonizers in infants. In fact, while the incidence of early onset neonatal GBS sepsis has decreased in the era of prophylactic antibiotic administration, sepsis with organisms highly susceptible to antibiotic resistance, such as *Escherichia coli*, and late onset sepsis has stayed the same or increased (Bauserman et al. 2013). Concerns regarding development of antibiotic resistant causes of neonatal sepsis has led some researchers to investigate the use of molecular methods for identification of women for

targeted intervention, and to the development of GBS vaccine (Brown and Denison 2018).

Offspring exposed to antibiotics before delivery and then fostered with unexposed dams had bacterial colonization similar to their unexposed foster siblings. The process by which an infant acquires bacteria is a subject of great interest because of the potential of preventing or correcting dysbiosis before suboptimal outcomes can occur. Infants born by cesarean section are colonized with more skin and environmental bacteria than those born vaginally (Dominguez-Bello et al. 2016; Bäckhed et al. 2004). Infants receiving formula feedings or antibiotics are at even greater risk of colonization with obesogenic or immune-reactive bacteria (Yasmin et al. 2017; Bokulich et al. 2016). Mice and humans are initially colonized with facultative anaerobes that soon give way to strict anaerobes that colonize the gastrointestinal tract (Jost et al. 2012). The adult pattern of colonization is acquired by 2-3 years of life in humans and 18 days in mice (Hill et al. 1990). Though neonatal physiology and milk diet supports bacterial niche specialists that differ from the usual adult colonizers, the early colonization process is linked to later growth and immune programming (Nakagaki et al. 2018; Milani et al. 2017). Conflicting data exist regarding the heritability of microbiota; however, the influence of diet, environment, and physical contact are known to be significant. More research is indicated to unravel the effects of local environment, genetic propensity, and diet on microbiota formation as each of these offers a vehicle to influence programing during the critical window of development. In these experiments, the dams that received antibiotics influenced offspring and foster offspring colonization either as a result of antibiotic residue transmitted through breastmilk, or from induced maternal dysbiosis resulting from

antibiotic administration. Antibiotic exposure via breastmilk seems unlikely as the breastmilk had no residual antibiotics by 7 postnatal days, and the antibiotics were delivered IP before delivery and would therefore represent a more direct and substantial dose that would cross the placenta. Control dams seeded normal colonization patterns in both natural and fostered offspring despite prenatal antibiotic exposure of the fostered pups leading to the supposition that the dysbiosis in the antibiotic-exposed groups originated from altered skin, oral, fecal, and breastmilk microbiota of treated dams.

Dams that received antibiotics during more than one pregnancy had offspring with higher abnormal bacterial counts and increased beta diversity compared to the offspring of dams with only one antibiotic exposure. There are currently no studies that examine the cumulative antibiotic exposure burden in mothers and the microbial colonization of their offspring. In humans, infants born of emergent cesarean sections and therefore exposed to more antibiotics suffered longer lasting dysbiosis than those exposed to prophylaxis of planned cesarean sections (Azad et al. 2016). Although the conditions which led to the need for urgent cesarean likely confounded results, it may be even more critical to re-establish a normal microbiota in mothers that require multiple antibiotic exposures before delivery.

Early dysbiosis continued to exert an influence on offspring microbiota for up to 30 weeks in our experiments even with a change from regular chow to a western diet that was high in sugar and fat and low in fiber. Though diet and sex differences both exerted strong influences on microbial selection, antibiotic exposure was still discernable in principal coordinate analysis at 29 weeks indicating that early pioneer bacteria change the microenvironment leading to prolonged effects on future colonizers.

Limitations of studying the microbiota in mice include the differences in diet, living conditions, anatomy, and genetics. The human vaginal microbiota differs from the murine vaginal microenvironment and is uniquely dominated by *Lactobacillus*, which are especially enriched during pregnancy (Romero et al. 2014; Miller et al. 2016). Mouse vaginal microbiota are dominated by Staphylococcus, Enterococcus, and Lactobacillus, which are not stable throughout the estrus cycle (Vrbanac et al. 2018). Early in murine pregnancy, fecal *Bifidobacterium* and *Akkermansia* increase in abundance then decrease as pregnancy progresses, while *Bacteroides* increases and remains elevated (Gohir et al. 2015). Human fecal microbiota changes dramatically during pregnancy and is characterized by increased abundance of Actinobacteria and Proteobacteria, that do not return to baseline for at least 6 weeks post-partum (Koren et al. 2012; MacIntyre et al. 2015). Both the human and mouse fecal microbiota are dominated by Bacteroidetes and Firmicutes, though not in the same ratio (Nagpal et al. 2018). Differences in microbiota between species are accompanied by some anatomical differences such as a bicornate uterus in mice and a vestigial cecum in humans, dietary differences, housing differences, and differences regarding control of environmental pathogens. Although care should be taken before translating murine microbiota studies directly to humans, the similarity of gross anatomy and microbial colonization between mice and humans allows for the strict control needed to elucidate important host-microbe interactions and mechanistic relationships which may be invaluable for validation studies in humans.

A limitation of sequence-based studies is that identified DNA may be from samples containing dead, dormant, or damaged bacteria. Taxonomic approaches based on 16s rDNA may distinguish microbial diversity and ecologic structure but cannot

determine function or causal relationships. The function of known bacteria is mostly determined by in vitro studies, which may not reveal the behavior of bacteria in their natural niche under the pressures of selection and competition. This study does reveal, however, that maternal vaginal and gastrointestinal microenvironments are altered even by brief exposure to both oral and injected antibiotics. Alterations to the maternal microbiota are transmitted to the offspring postnatally. Changes in early pioneer microbiota alter the long-term microbial colonization of offspring and are more severe in offspring whose mothers had more than one pregnancy with antibiotic exposure. Offspring exposed to antibiotics during gestation developed normal microbiota when reared by unexposed dams. More research is required to determine the mechanisms by which early colonizers are selected for offspring microbiota formation, in what way pathogenic bacteria might be removed without disturbing healthy microenvironments, and the nature of host-microbe interactions that influence growth and immune function throughout the lifespan.

Materials and methods (see Methodology Section)

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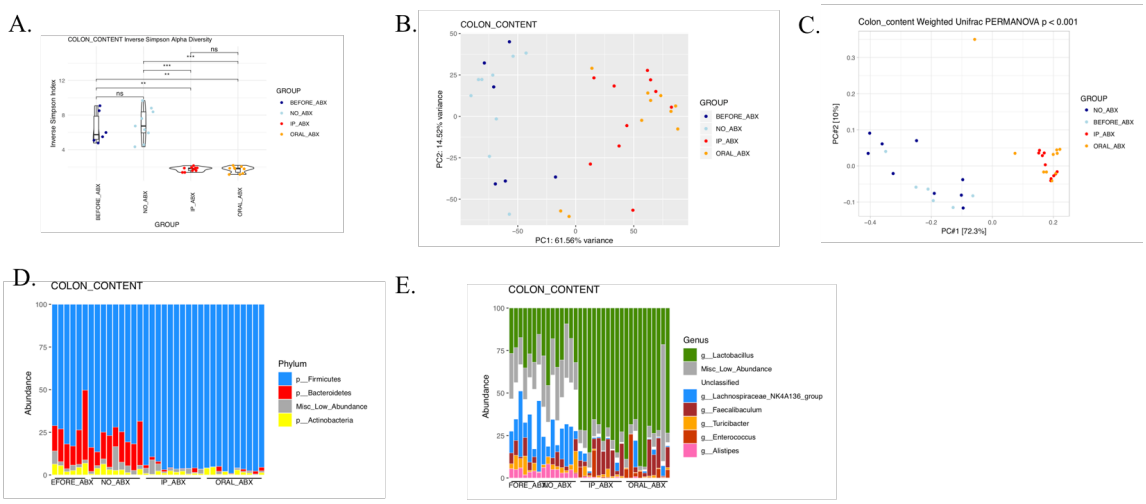

Figure 1. The composition of the fecal microbiota of pregnant mice is altered by both oral and intraperitoneally injected antibiotics during the three days prior to delivery. (A) Alpha Diversity using Inverse Simpson (B) Principal components analysis (PCA) of unweighted UniFrac distance matrix ($p < 0.002$) and (C) Weighted UniFrac distance matrix. (D) Relative abundance of colonic bacterial phyla before and after maternal antibiotic exposure. Unclassified sequences were grouped together under the label "miscellaneous low abundance." (E) Relative abundance of bacterial genera before and after maternal antibiotic exposure. Genera with an average relative abundance below 1% are grouped together and labeled "miscellaneous low abundance." Significant differences in beta diversity were calculated using Permanova with a significance value of $p \leq 0.05$. Figures generated using R.

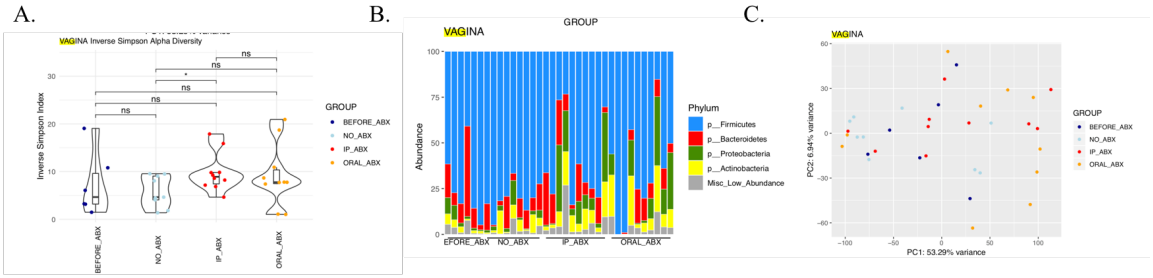

Figure 2. There was low bacterial abundance in the vagina of pregnant dams at delivery, but alpha diversity was slightly increased in dams that received i.p. antibiotics (A). Dams that received antibiotics prior to delivery had increased *Protoebacteria* that was not statistically significant (B). There was minimal clustering in the vaginal microbiota based on antibiotic exposure (C.)

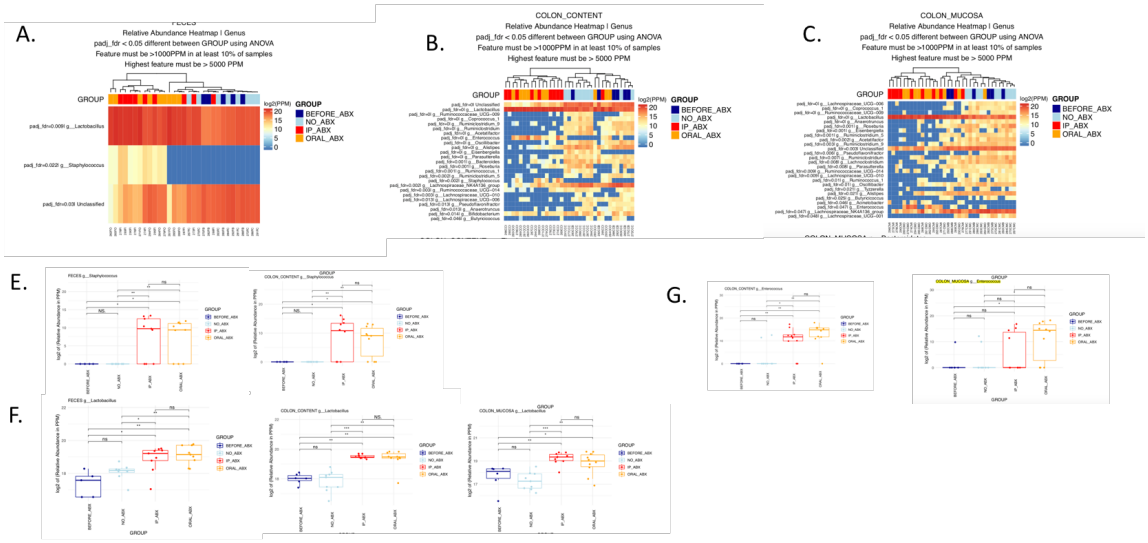

Figure 3. Heatmap of the relative abundance of significant differences between groups by antibiotic treatment. Bacteria must be in >10% of samples and at least 1000 parts per million. Significance is determined by ANOVA with adjusted p values > 0.05. Difference in Fecal samples (A), Colon content samples (B), and colonic mucosa samples (C). Relative abundance differences of *Staphylococcus* in feces and colon content (E). Relative abundance of *Lactobacillus* in feces, colon content, and colonic mucosa (F). Relative abundance differences of *Enterococcus* in colon content and mucosa (G). Boxplots derived in R using Mann Whitney with significance of p< 0.05.

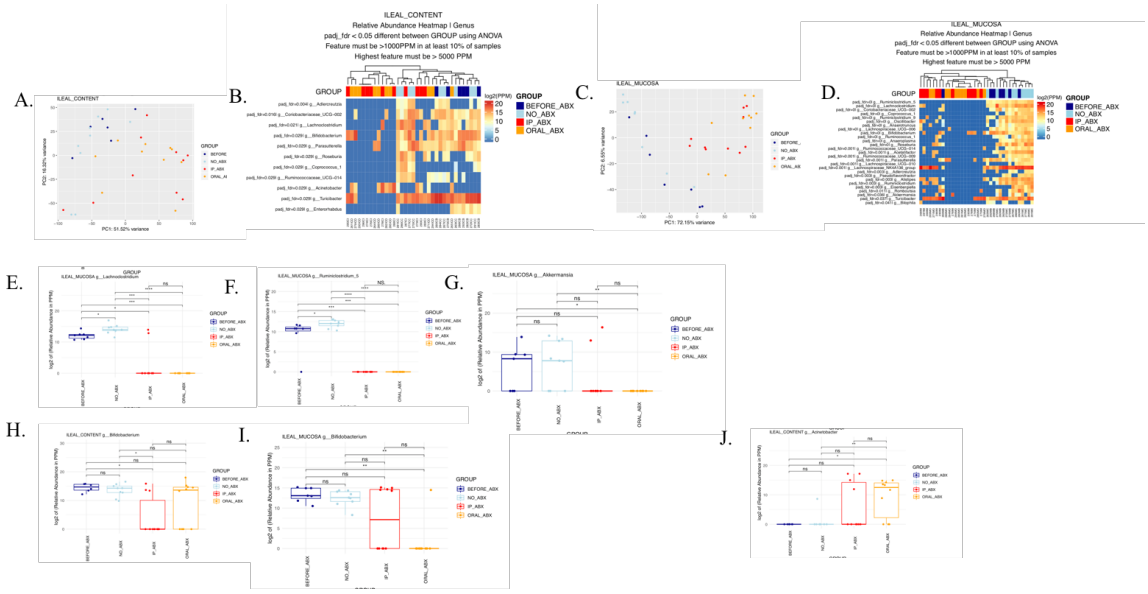

Figure 4. The composition of the ileal microbiota of pregnant mice is altered by both oral and intraperitoneally injected antibiotics during the three days prior to delivery. (A) Principal components analysis (PCA) of unweighted UniFrac distance matrix of ileal contents (B) Heat map of significant relative abundance differences in ileal contents. (C) PCA of ileal mucosa (D) Heat map of significant differences in relative abundance of ileal mucosa. (E-G) Boxplots of differences in the relative abundance of selected genera. (H-I) Differences in the relative abundance of Bifidobacterium in ileal content and mucosa. (J) Relative abundance of Acinetobacter. Significant differences in beta diversity were calculated using Permanova with a significance value of p≤0.05. Adjusted p values in heatmaps and boxplots were calculated using Wilcoxon Rank Sum with a significance value of p <0.05.

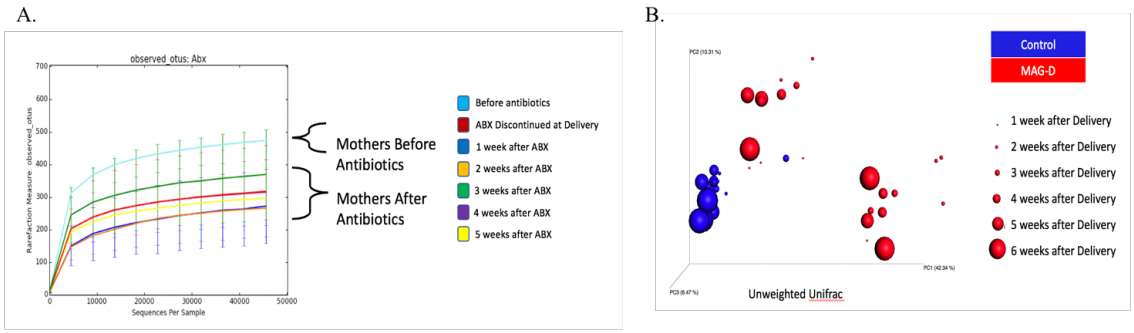

Figure 5. Dams that received antepartum antibiotics had reduced fecal alpha diversity and increased fecal beta diversity throughout the offspring developmental period. (A) Observed operational taxonomic units (OTUs) of fecal microbiota sequenced weekly using 16s rDNA. (B) Maternal fecal microbiota weekly from delivery. Principal components analysis (PCA) of unweighted UniFrac distance matrix. Graphs constructed using Qiime2.

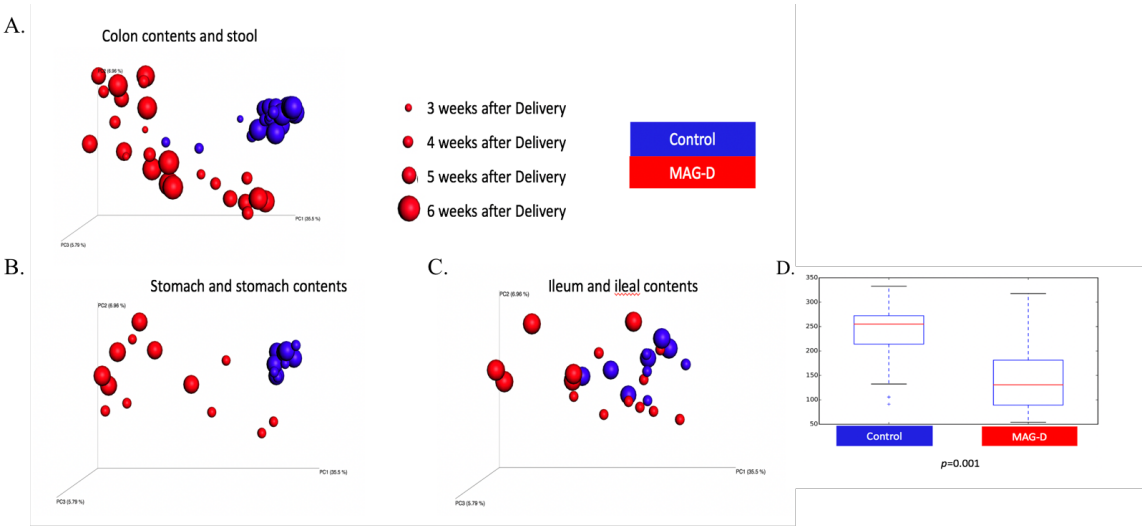

Figure 6. Offspring had decreased alpha diversity and increased beta diversity if exposed to antibiotics prior to delivery. Principal component analysis of fecal beta diversity using unweighted UniFrac analysis of the (A) colon and colon contents, (B) stomach and stomach contents, and (C) Ileum and ileal contents. Alpha diversity (D) using Inverse Simpson, $p<0.001$, Mann Whitney). Figures constructed using Qiime 2. Oral antibiotics show similar beta diversity relative to controls.

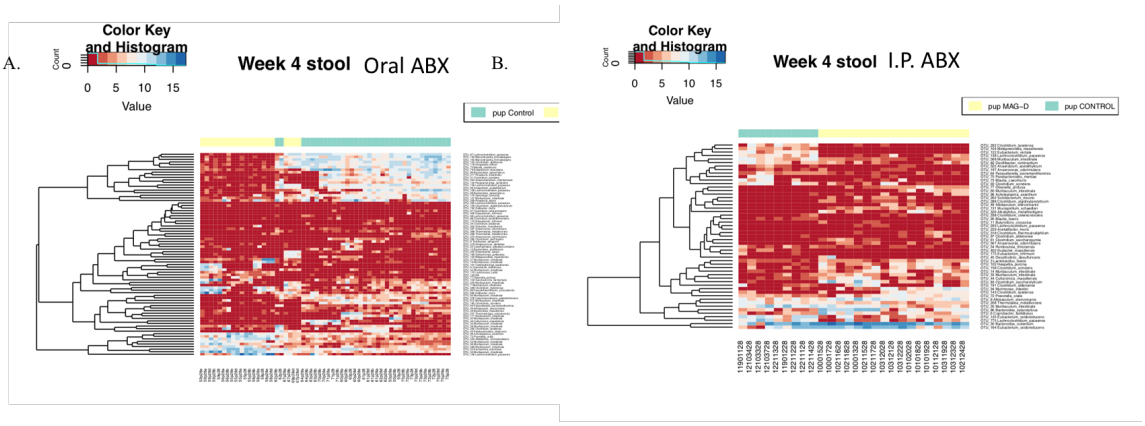

Figure 7. Unsupervised clustering of bacterial populations identified by sequencing the 16s rRNA gene in the feces of 4-week-old mice exposed to (A) oral antibiotics (vancomycin, neomycin, doripenem) in water during the last week of gestation, or intraperitoneal antibiotics (vancomycin, gentamicin, piperacillin-tazobactam) given daily for 3 days prior to delivery.

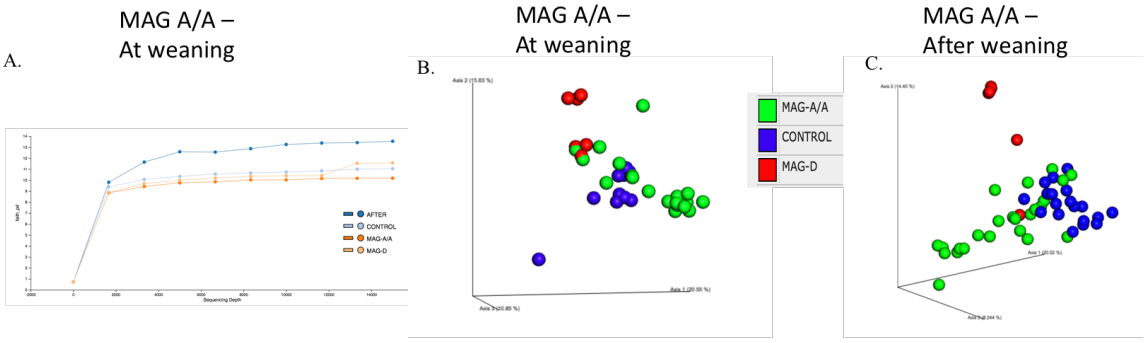

Figure 8. Frequently prescribed prophylactic antibiotics, ampicillin, 100mg/kg, and Ancef, 60mg/kg, (MAG A/A) were administered to pregnant C57BL/6J dams daily intraperitoneally during days 18-20 of gestation. (A) At 3 weeks, offspring had similar fecal alpha diversity, measured using faith_pd, in comparison to control offspring and slightly higher diversity than those receiving more broad spectrum infected antibiotics (vancomycin 15mg/kg, gentamicin 4mg/kg, and piperacillin-tazobactam 125mg/kg (MAG-D). (B) Offspring whose dams received MAG A/A and MAGD clustered separately at weaning and (C) 1.5 weeks after weaning using unweighted UniFrac analysis of beta diversity. Figures produced using Qiime2.

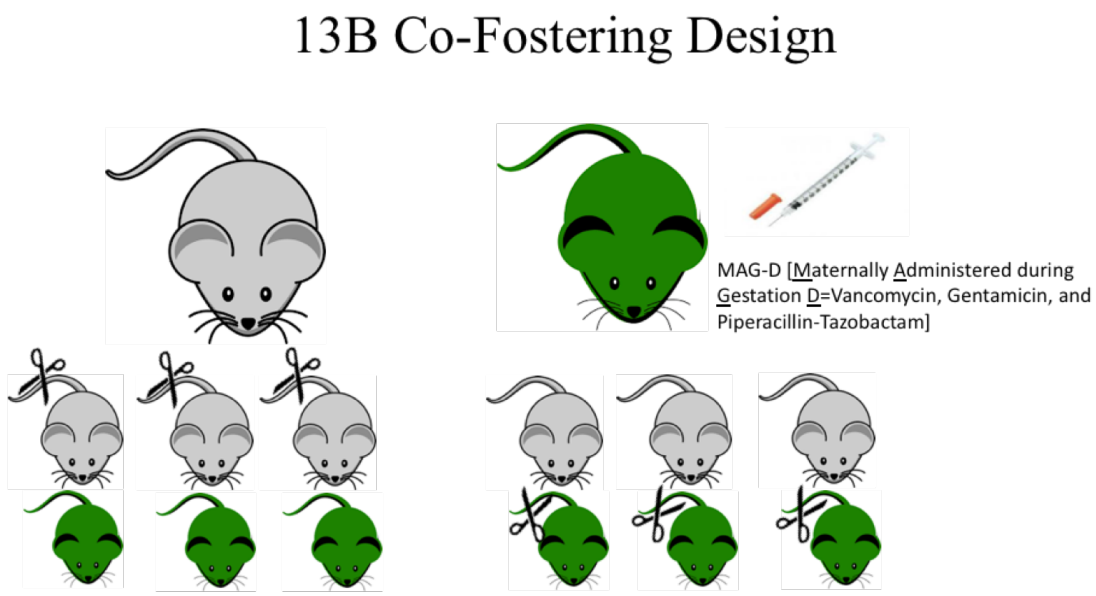

Figure 9. Co-fostering Experimental Design. C57Bl/6NCr pregnant mice were given normal saline or vancomycin 15mg/kg, gentamicin 4mg/kg, or piperacillin-tazobactam 125mg/kg intraperitoneally daily on gestational days 18, 19, and 20 (dosing weights based on pre-pregnancy weights of 20g). On day 3-5 of life, ½ of each litter was fostered with a mother in the opposite group. Remaining offspring tails were snipped for later identification.

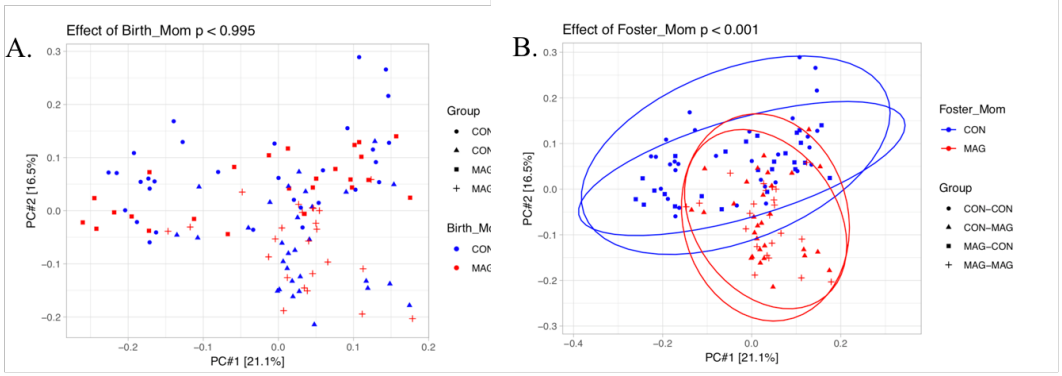

Figure 10. Vancomycin, gentamicin, and piperacillin-tazobactam were administered intraperitoneally to C57BL/6 dams for up to 3 days prior to delivery. Offspring were divided and exchanged at 3-5 days of life. Co-fostered offspring had similar microbiota to foster siblings regardless of prenatal antibiotic exposure (A). Offspring exposed to antibiotics prior to delivery and fostered with unexposed dams had microbiota similar to foster siblings, while offspring of dams not exposed to antenatal antibiotics had microbiota similar to foster siblings reared by exposed dams (B). Principle component analysis of unweighted UniFrac distance matrixes. Statistical differences were determined using PERMANOVA with significance values of $p < 0.05$. (con-con=born to control, reared by control; con-mag=born to control, reared by dam with maternal antibiotics during gestation (MAG); mag-con=born to dam with MAG, reared by control; mag-mag=born to MAG dam, reared by MAG dam)

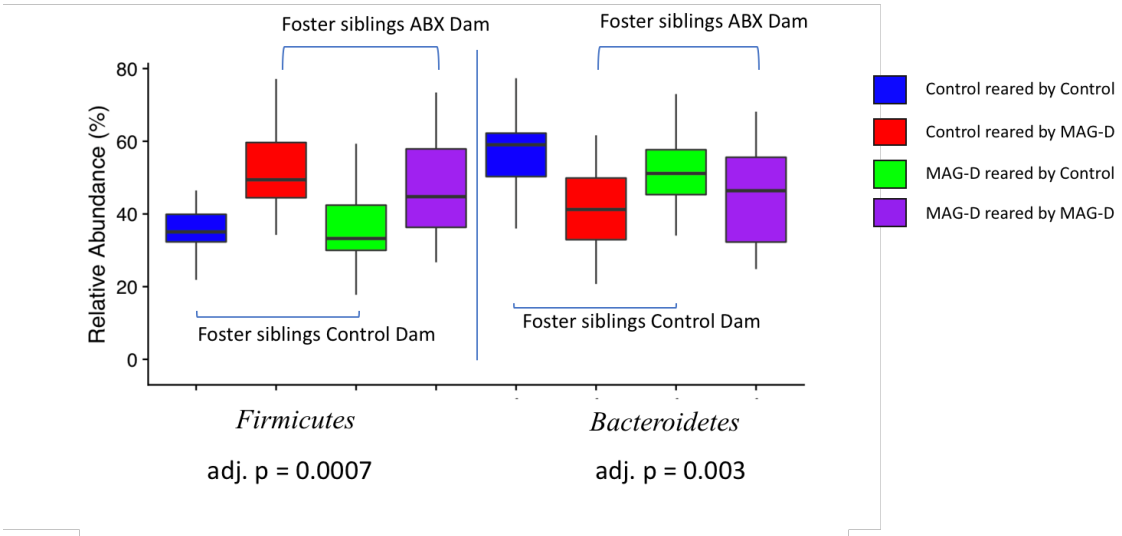

Figure 11. Offspring reared with dams that received no antibiotics prior to delivery have higher *Bacteroidetes* and lower *Firmicutes* at weaning than their siblings reared with antibiotic-treated dams. Adjusted p values were calculated using Wilcoxon Rank Sum with a significance value of p <0.05.

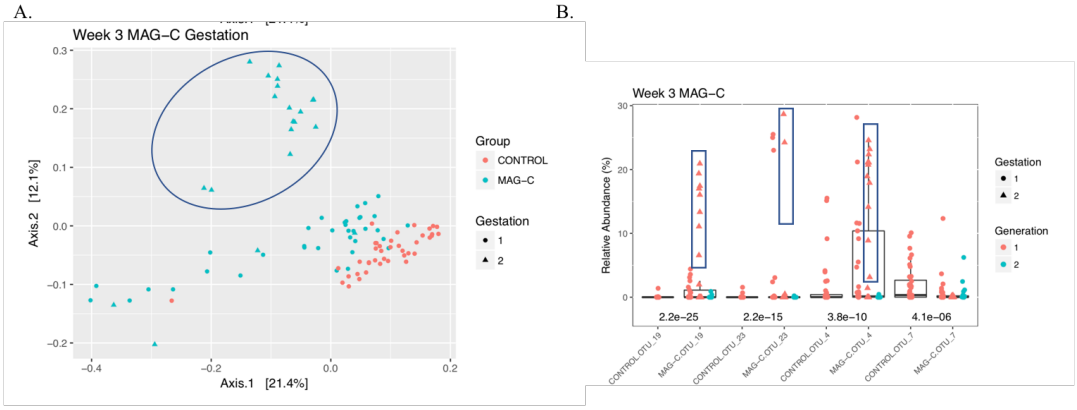

Figure 12. C57Bl/6NCR were given vancomycin, neomycin, and doripenem in drinking water x 7 days prior to delivery. Offspring from the dam's second pregnancy with antibiotic exposure clustered separately from control offspring and offspring of dams with a single exposure to antibiotics before delivery (A). Fecal beta diversity measured using unweighted UniFrac. Figures generated using R.

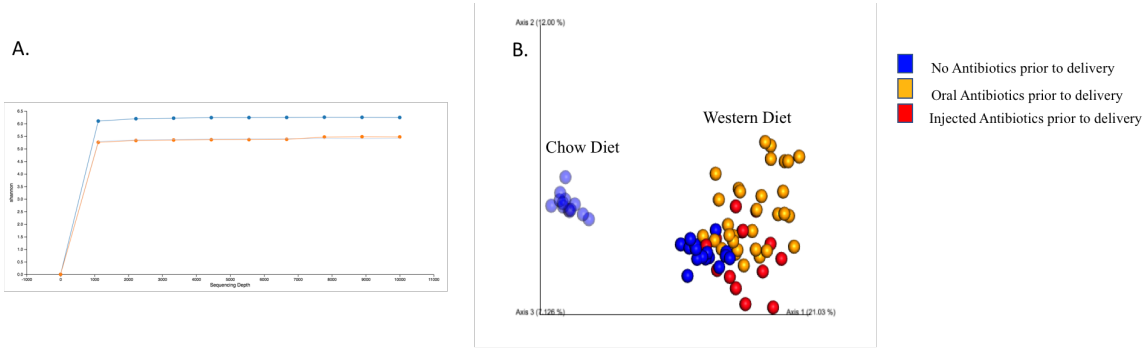

Figure 13. At 30 weeks of age, offspring had decreased alpha diversity and increased beta diversity if exposed to antibiotics prior to delivery. (A) Principal component analysis using unweighted UniFrac of fecal beta diversity. Alpha diversity (B) using Shannon Diversity Index. Figures constructed using Qiime 2.

Offspring Growth in the Aftermath of Maternal Intrapartum Antibiotic Prophylaxis

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Running title: Growth after IAP

Abstract: Prophylactic antibiotics are administered to nearly half of all pregnant women prior to delivery to prevent neonatal early onset sepsis and surgical site infection. Intermittent and long-term oral antibiotic exposure is associated with obesity in children and adults, but the effects of brief intravenous antibiotics like those used for maternal surgical and sepsis prophylaxis are not well studied. In this study, we administered parenteral antibiotics to pregnant C57Bl/6 mouse dams prior to delivery and placed the offspring on a western diet at weaning to elucidate the long-term effects of injected antibiotics on the intestinal microbiota and the repercussions of altered colonization on growth. Offspring had disrupted bacterial colonization that lasted through maturity. Metagenomic analysis revealed that genes encoding protein functional units remained altered in female adult offspring of exposed dams. Females had increased obesity, decreased glucose tolerance, and insulin insensitivity if their dams had antibiotics prior to delivery. Both males and females had increased liver steatosis and low-grade focal liver inflammation. There was no change in intestinal gene expression in response to altered bacterial colonization, but significant up-regulation in intestinal heat shock proteins in response to the western diet. Liver gene expression was changed in female offspring of antibiotic-exposed dams.

Key Words: **IAP, Obesity, Antibiotics, Microbiota, Pregnancy**

Nearly half of all full-term, healthy newborns are exposed to antibiotics during late gestation to prevent early onset sepsis from maternal Group B *Streptococcus* (GBS)

colonization or to prevent maternal surgical site infection during cesarean section (Verani et al. 2010; Committee on Practice Bulletins-Obstetrics 2018). Though intrapartum antibiotic prophylaxis (IAP) has reduced the incidence of neonatal early onset sepsis, the incidence of late onset sepsis remains unchanged (Schrag and Verani 2013). Sepsis from gram negative or antibiotic resistant strains has also increased in the era of prophylaxis (Bauserman et al. 2013; Stokholm et al. 2014; Simonsen et al. 2014). Oral antibiotics administered during gestation, infancy and childhood have been linked to adverse outcomes such as obesity (Korpela and de Vos 2016; Cox and Blaser 2015), allergy (Ahmadizar et al. 2018), inflammatory bowel disease (Örtqvist et al. 2019), and impaired social behavior (Leclercq et al. 2017). Though changes to the infant microbiota have been observed after IAP (Nogacka et al. 2017; Azad et al. 2016; Corvaglia et al. 2016), which consists of intravenous antibiotics of short duration, little research has focused on the long-term outcomes associated with disruption of the microbiota as a result of IAP during this critical period of infant colonization.

The timing of initial bacterial colonization is currently under debate. The traditional dogma and evidence from nearly 70 years of germ-free animal research have supported the “sterile womb” theory, but recent molecular-based studies have indicated that the first bacterial contact may occur in utero (Perez-Muñoz et al. 2017; Walker et al. 2017). Until direct evidence of bacterial transfer in utero is obtained, the question is likely to remain unresolved. Whether first contact occurs during late gestation or during the birth process, the sterile or nearly sterile infant is immediately exposed at delivery to bacteria occupying every conceivable niche. Bacterial colonization of the newborn varies

depending on many factors including maternal genetics, culture, geographic area, water supply, medication, delivery method, breastfeeding, and hygiene. As the newborn physiology matures and stabilizes, and feedings change from primarily milk to the adult pattern over time, bacterial populations gradually change toward an adult-like bacterial consortium by about 3 years of age (Yatsunenko et al. 2012).

Bacteria interact with the host during this critical developmental window to influence metabolic, immune, and neurobehavioral programming (Cox et al. 2014; den Besten et al. 2013; Diaz Heijtz 2016). Bacteria may interact with the host via direct communication with adjacent cells by occupying niches such as the mucosa that are frequently sampled and transported by mobile immune cells or amenable to extracellular signaling by way of excreted substances (Perez-Pascual et al. 2016; Yu et al. 2018; Goto and Ivanov 2013). Luminal bacteria ferment otherwise indigestible substances that are then absorbed supporting growth, immunity, and behavior (Brestoff and Artis 2013). Understanding the impact of limited doses of intravenous antibiotics administered to the mother prior to delivery on the neonatal colonizing species, and the implications of changes to these early founding populations on later colonization and on immune and metabolic programming may be crucial to the long-term health of the neonate. Therapeutic targets and timepoints may be identified to maximize recolonization with desirable species or consortiums to promote optimal growth, metabolism, and immune response when antibiotic administration is required for the safety of the mother or infant.

In this study we administered intraperitoneal antibiotics to C57Bl/6 pregnant mice during the last days of their pregnancy in order to determine if exposure to injected

antibiotics altered the growth and energy metabolism of offspring. We then challenged the offspring with a Western diet that matched the CDC documented macronutrient content of the citizens of the United States from 2000-2008 (50% carbohydrates (15% fructose), 15% protein, 35% fat (11% saturated)) (Wright and Wang 2010). Changes in the fecal microbiota, weight, energy consumption, glucose tolerance, and insulin levels were monitored weekly for 30 weeks (Fig. 1).

Materials and Methods: (See Methodology Section)

Results

Dams and their offspring have altered colonization and decreased bacterial richness in observed taxonomic features after exposure to limited doses of antibiotics prior to delivery.

Pregnant female dams were given vancomycin, gentamicin, and piperacillin-tazobactam orally or IP daily from day 18 to day 21 of gestation. Antibiotics were discontinued at birth. Dams had reduced fecal bacterial richness and altered colonization at delivery (Fig 2A, B). Offspring were placed on the western diet at weaning. Feces from male and female offspring was collected at weaning and at harvest for 16s rDNA analysis. At weaning, offspring of dams that were exposed to antepartum antibiotics had reduced fecal alpha diversity and bacterial colonization that differed from control litters (Fig 2C, D).

A subset of fecal DNA from female offspring was analyzed by shotgun sequencing to elucidate bacterial functional capacity after late gestational antibiotic exposure. Shotgun sequencing confirmed reduced protein functional genetic pathways in offspring of antibiotic treated dams as measured by bacterial taxonomic feature count, but there was not a difference in protein functional units if measured by the Inverse Simpson, a weighted measure less sensitive to rare species (Fig. 3A-E). *Mucispirillum schaedleri*; *Parabacteroides johnsonii, merdae, and distonsis; Bacteriodes vulgatus; and Lachnospiraceae_bacterium* are significantly reduced in the female offspring of dams given IP antibiotics prior to delivery while *Akkermansia muciniphilia*; *Alistipes*, *unclassified*; *Parabacteroides goldsteinii*; and *Butyrivibrio crossotus,* unclassified are increased (4A). Genes coding for 20 bacterial enzymes and decreased while 5 are significantly increased in antibiotic exposed progeny (4B). Genes encoding for 46 protein products are decreased while 5 are increased (4C). Bacterial colonies, enzyme codes, and protein products all cluster differently in animals whose dams received IP antibiotics before birth (Fig 4D-F)

Female offspring whose mothers received IP antibiotics had increased weight gain, glucose intolerance and insulin insensitivity on the western diet

Offspring were weighed weekly for 30 weeks. Female offspring were significantly heavier if their dams received IP antibiotics prior to delivery. All male offspring became obese in response to the western diet, with no significant differences in weight as a result of prenatal antibiotic exposure (Fig. 5A, B). Glucose tolerance tests

were performed every 5 weeks after placement on the western diet. Offspring exposed to antenatal antibiotics had elevated glucose and insulin levels during the glucose tolerance test (Fig 5 C-F).

Offspring exposed to antenatal IP antibiotics prior to delivery had increased liver steatosis and inflammation at 29 weeks of life.

Offspring were sacrificed at 29 weeks of age. Males and females had increased liver weights if their dams received antenatal IP antibiotics prior to delivery. There was no difference in the weight of fat in the mesenteric fat compartment. There was no difference in the gastrocnemius muscle weight (Fig 6A, B). Liver sections stained with hematoxylin and eosin revealed increased steatosis and mild inflammation in offspring of dams that received antenatal antibiotics (Fig 6C-F).

Female offspring had liver gene expression changes at 29 weeks of age on the western diet after prenatal exposure to IP antibiotics.

RNA was extracted from the liver tissue of female mice on the western diet that were sacrificed at 29 weeks of age. RNAseq revealed expression differences in the liver tissue in mice whose dams had received prenatal antibiotics (Fig 7A). There were 65 upregulated or down regulated genes in the livers of adult female mice that met the criteria of significance ($p< 0.05$ and log fold change >2) (Fig 7B). The top 5 genes that were differentially expressed between offspring of dams given IP or no antibiotics were *Mafb* and *Ddit4,* which were significantly down regulated, and *Id3, Elov7, Cxcl10,* which

were significantly upregulated ($p<10e-4$, FDR<0.15, n=16) (Fig 7C). Males on the western diet had 748 expression differences between offspring of dams that did or did not receive prenatal IP antibiotics ($p<0.05$ and log fold change > 2, all FDR>0.5) (data not shown). There were 194 genes with expression differences that met the criteria of $p<0.05$, log fold change >2, and FDR<0.05 between mice on the regular chow diet and those on the western diet (data not shown). There were 377 genes with expression differences that met the criteria of $p<0.05$, log fold change >2, and FDR< 0.05 (data not shown).

Dietary changes have a greater effect on offspring gastrointestinal gene expression than prenatal antibiotic exposure.

RNA was extracted from samples from the ileum and colon of offspring on the western and regular chow diet, whose dams had or had not received antibiotics prior to delivery. The western diet elicited significant up-regulation in the expression of heat shock proteins *HSPA1A* and *HSPA1B,* and the down-regulation of *CYFIP2 (*adj. p <0.01*)* (Fig 8A). There were no significant differences between the gastrointestinal gene expression of mice exposed to IP antibiotics prior to delivery and those not exposed (Fig 8B).

Discussion

Obstetric and newborn care has been revolutionized by the availability of antibiotics. Before they were readily available, any time in an institutional setting such as

a jail, poorhouse, asylum, or hospital increased the risk of mortality, especially for childbearing women and children. Neonatal intensive care began in earnest only after antibiotics were being used regularly. The incidence of early onset neonatal sepsis has decreased since prophylaxis for all women colonized by GBS. Likewise, surgical sepsis has decreased since prophylaxis began. Perhaps we have become complacent about this practice of giving a few doses of antibiotics, thinking that only good can come of it. Similar thoughts have occurred throughout the history of medical care. At one time, we thought it prudent to give oxygen into the incubators of all premature newborns and synthetic estrogen to women with miscarriages. The difficulty with some therapy is that it works, at least in the short term. It is sometimes difficult to identify subtle long-term effects, when the short-term benefits seem obvious. We must treat nearly 100 women prophylactically in order to prevent 1 case of neonatal early onset sepsis. We owe it to the other 99 infants to understand the risks they might face as a result before accepting that this is the best means of prevention available.

In this study pregnant dams had altered fecal microbial colonization at delivery and throughout the offspring's developmental period if exposed to either oral or injected antibiotics. The offspring had reduced bacterial abundance and altered colonization if reared by a mother so exposed. Interestingly, metagenomic analysis of alpha diversity revealed that though the observed number of bacterial genes coding for enzyme and protein pathways were reduced significantly in this cohort, the weighted measures were not significantly different. This likely shows that full colonization with all available species provides multiple layers of pathway redundancy, thus giving the environment

some resiliency against transient changes. The danger of antibiotic exposure then may not be just the initial killing within the environment but limiting the ability of the microbes present from recovering from future disturbances to the microbial milieu.

By weaning, pups colonize with distinctly altered colonization patterns if their mother had a short course of antibiotics. Species especially susceptible to injected antibiotics such as *Mucispirillum shaedleri* that is usually an abundant inhabitant of the mucus layer in rodents (Loy et al. 2017), were obliterated early and never recovered. *Alistipes*, common in the mammalian intestine, and associated with high fat diet and inflammatory bowel disease was able to flourish (Daniel et al. 2014; Schirmer et al. 2018). Some strains of *Alistipes* are resistant to vancomycin and gentamicin, along with several other antibiotics (Hugon et al. 2013), and may have therefore had a competitive advantage. Early colonization with resistant species may be one byproduct of pre-delivery antibiotic exposure.

Changes in early colonization led to altered bacterial populations in adulthood and to distinct bacterial protein synthesis. Genes for different functional protein units such as for enzyme formation were significantly different in adult offspring whose dams received antenatal antibiotics. For example, genes encoding gamma-glutamyltransferase (GGT) significantly increased in the bacteria of animals receiving prenatal antibiotics. GGT is highly conserved across many species in the membranes of absorptive or secretive cells such as the liver in mammals. Elevated GGT levels in humans are indicative of hepatobiliary disease (Ruppin et al. 1982), but are also seen pancreatic disease (Carr-Locke and Davies 1980), heart disease (Jiang et al. 2013), and chronic obstructive

pulmonary disease (Kim et al. 2014). Bacterial GGT can act as a virulence factor by inducing inflammation and apoptosis in epithelial cells and by suppressing the immune response through inhibition of T-cell and dendrocyte activity (Ricci et al. 2014). GGT has been implicated in the development of hepatobiliary tract cancer (Boonyanugomol et al. 2012) and cancer metastasis by way of GGT-rich vesicles secreted from the outer membranes of bacteria or from eukaryotic exosomes (Franzini et al. 2014; Kawakami et al. 2017). GGT of bacterial origin may be absorbed and delivered to host lymphatics and circulation allowing immunomodulation, easing bacterial translocation, and contributing to inflammation at distant sites. As the liver is the recipient of blood returning from the intestines via the portal vein, it may be the most likely destination of translocated bacteria and immunoactive bacterial products.

Intestinal expression of heat shock proteins, *HSPA1A* and *HSPA1B* were significantly upregulated in mice receiving the western diet, though there were no significant differences in intestinal gene expression by antibiotic exposure. The combination of stress induced by the western diet and decreased resilience provoked by maternal antibiotic exposure may have increased the likelihood of local inflammation and translocation with bacterial species equipped with genetic machinery to construct proteins such as GGT to maximize their competitive advantage. Elevated GGT levels are associated with a higher risk of type 2 diabetes, especially in women (Ahn et al. 2014), and predict reduced glucose tolerance and insulin resistance in healthy adults (Thamer et al. 2005). In this study, female offspring on the western diet with bacterial colonization

that overrepresented the genes for GGT manufacture were more obese, glucose intolerant, and insulin resistant than controls with normal bacterial colonization.

Females had increased steatosis and low-grade focal liver inflammation when exposed to perinatal antibiotics. Benign steatosis progresses to steatohepatitis as a consequence of elevated free fatty acids and oxidative stress (Naik et al. 2013). RNA was analyzed from obese female offspring liver to determine expression differences as a result of perinatal antibiotic exposure. Expression of *Mafb, Elovl7,* and *CXCL10* were upregulated in the livers of antibiotic-exposed obese female offspring compared to obese female control offspring. *Mafb* is a proinflammatory marker in white adipose tissue contributed by infiltrating monocytes and macrophages (Pettersson et al. 2015). *Elovl7* is an enzyme necessary for the very long chain fatty acid (VLCFA) elongation and can be controlled by invading viruses in order to maximize viral replication (Purdy et al. 2015). Elevated *CXCL10* induces steatohepatitis via inflammatory molecules and oxidative stress, contributes to hepatic cellular apoptosis, and is an independent risk factor for non-alcoholic steatohepatitis in humans and mice (Zhang et al. 2014). Antibiotic exposure prior to delivery was associated with the progression of benign steatosis toward steatohepatitis in offspring on a western diet, though whether this occurred as a result of altered metabolic programming during development or as a result direct interaction with translocated bacteria or viruses could not be determined.

Both males and females in the antenatal antibiotic group had small focal inflammation in the liver and increased steatosis compared to controls, but all males on the western diet were obese, glucose intolerant, and insulin resistant without regard to

prenatal antibiotic exposure. Perhaps the stress induced by the western diet created conditions for dysbiosis that superseded the influence of antibiotic exposure. Clearly the relationship between the microbiota, diet, and metabolic disease is complex and multifactorial.

This study confirms that limited doses of parenteral antibiotics administered prior to delivery does impact the initial commensal colonization in offspring, and that these changes last into adulthood. Decreases in bacterial richness lead to decreased resiliency in the bacterial population by reducing number of genetic pathways available for functional protein group formation. Changes in the host that induce local stress, such as the western diet, challenge the bacterial ecosystem possibly leading to opportunistic invasion by species equipped with virulence factors such as GGT that promote bacterial translocation and downstream inflammation. Additionally, altered colonization during critical developmental periods may alter growth or immune programming.

The sample size in this pilot study included 20 males and 20 females that received antibiotics prior to delivery; 10 mice of each sex were placed on the western diet, and 10 mice of each sex were placed on the regular chow diet. A matching number of mice with no antibiotic exposure made up control groups. A subset of these mice was euthanized at 10 weeks, and the remainder were euthanized at 29 weeks. This left only 8 mice per group for comparisons at the end of the study. Though there were statistically significant differences, the study should be repeated with larger numbers to confirm the associations and perhaps elucidate the mechanisms responsible for phenotypic changes resulting from differences in the microbiota.

Care should be taken when using animal models to infer meaning to human subjects. Mice, though similar to humans in many ways, and have a long history as a model species for human experimentation, have fundamentally different diets, including coprophagy, that profoundly impact the microbiota. The advantage of murine models for microbiota study is the extreme control that can be placed on genetics, environment, and diet. This experiment was conducted in a specific pathogen free environment that controls for common viruses and parasites. The mouse diets and water were irradiated before consumption, and the dams were littermates. As the microbial environment is so diverse and variable, these controls lend considerable stability and yield valuable information for confirmation in human studies where confounding variables are difficult to control.

We do not propose, here, that a single bacteria or bacterial consortium is responsible for the obesity, metabolic changes, or inflammation that characterizes the phenotype of our antibiotic-exposed mice. More research is needed to elucidate the mechanisms that would facilitate those changes. We do, however, propose that the use of prophylactic antibiotics be carefully considered in light of the possible long-term consequences to otherwise healthy, term neonates. Providers should question their sense that short-course parenteral antibiotics will have minimal if any effect on the newborn. Until we learn the outcome of changes at this critical time of microbiota establishment, or how to mitigate changes by targeted restoration, effort should be expended to treat only those that will most benefit with risk-based narrow spectrum antibiotics. Real-time identification of GBS such as by molecular measures at the time of delivery should

replace the standard culture-based identification at 37 weeks of gestation. GBS vaccine development should be prioritized.

Contributions:

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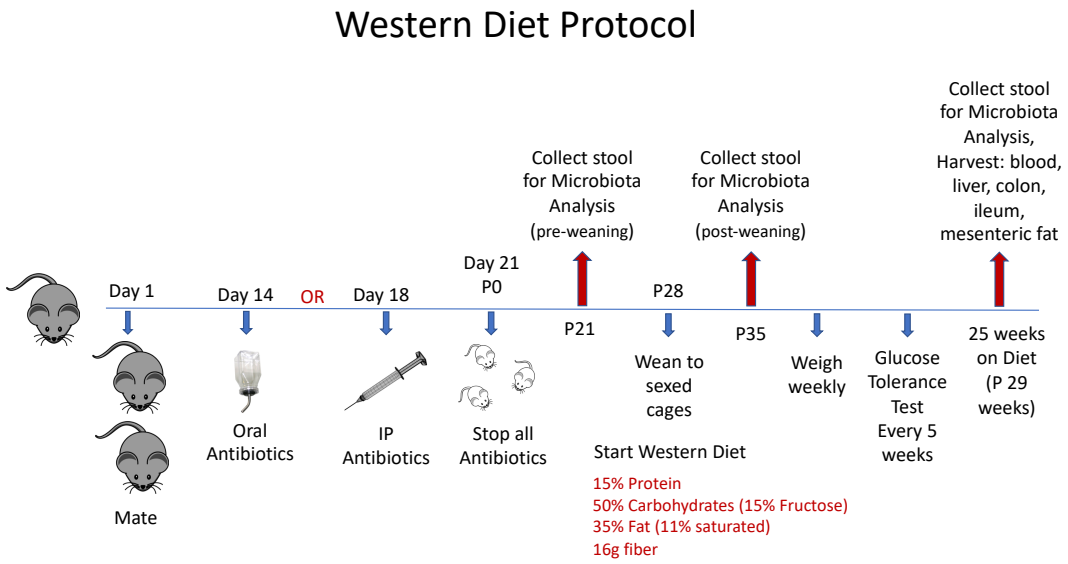


Figure 1. C57Bl/6-NCR female age-matched or littermates were mated to age-matched littermate males. After 3 days, males were removed. At 14 days pregnant dams randomized to the oral group received 500mg/L vancomycin, 5ml/L neomycin, 125mg/L doripenem in drinking water until delivery. At 18 days pregnant dams randomized to the intraperitoneal (IP) group received 15mg/kg vancomycin, 4mg/kg gentamicin, and 125mg/kg piperacillin-tazobactam daily until delivery. Offspring were weaned at 4 weeks to single sex cages and placed on either a regular chow or western diet. Stool was collected for 16s rDNA sequencing at weaning and at harvest. Glucose and insulin levels were monitored every 5 weeks. Liver collected for H & E staining and RNAseq. Female stool at weaning and at harvest was shotgun sequenced.

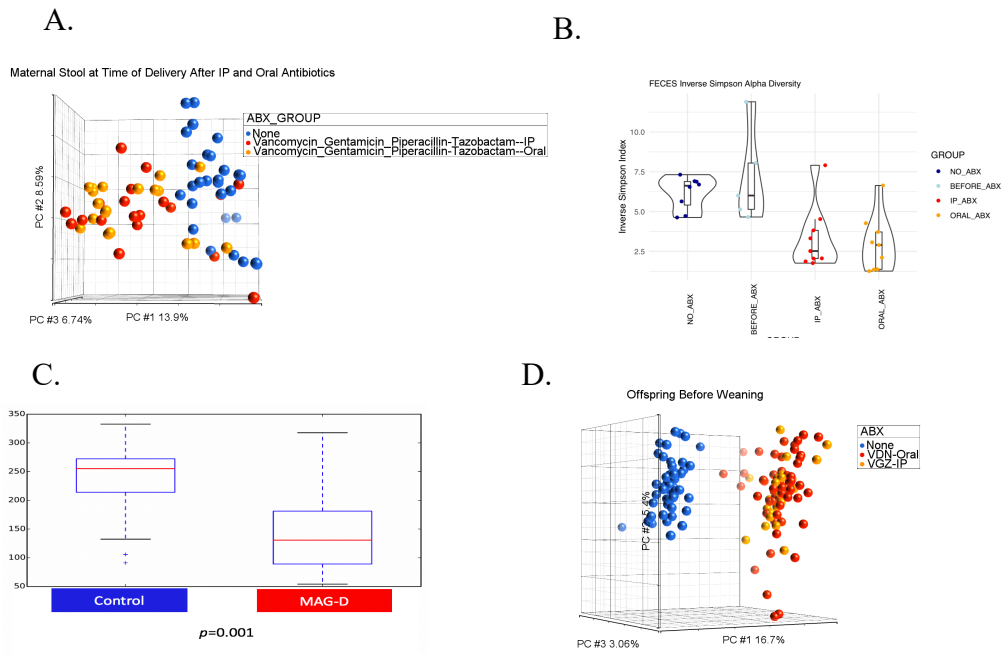


Figure 2. C57Bl/6 pregnant females received Vancomycin, Gentamicin, and Piperacillin-Tazobactam for 2 days prior to delivery. Dams had altered fecal bacterial colonization at the time of offspring delivery (A). Maternal fecal alpha diversity was reduced at the time of delivery (B). At weaning, offspring whose mothers were exposed to antibiotics prior to delivery had reduced alpha diversity (C) and altered colonization (D). Alpha diversity was measured by the inverse Simpson index, and beta diversity was measured using unweighted principle coordinate analysis.

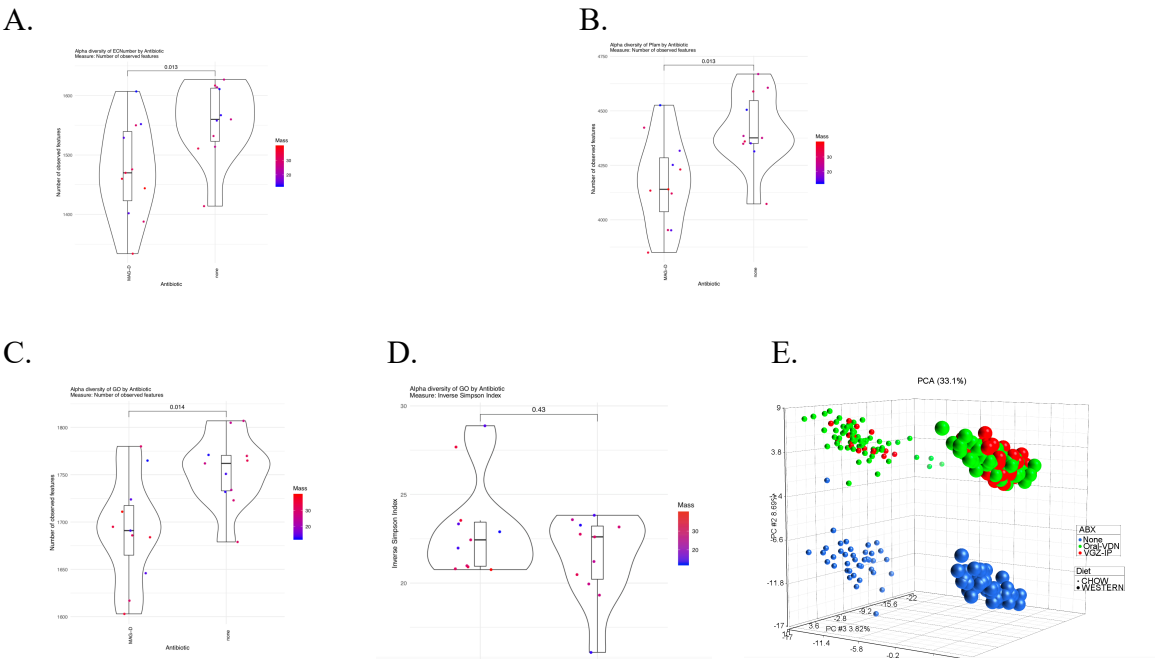


Figure 3. Functional protein groups such as enzymes (A), functional protein families (B), and proteins classified by GO terms (C) are reduced as measured from observed taxonomic features in shotgun sequencing, but not significantly different when measured using Inverse Simpson (D). (Visualizations A-D generated by JAMS, McCulloch et al, unpublished) Offspring receiving both the Chow diet and the Western diet remain uniquely colonized at 30 weeks of life after their dams received brief prenatal antibiotic exposure (E). Principal coordinate analysis determined by ANOVA. Visualizations generated in PARTEK.

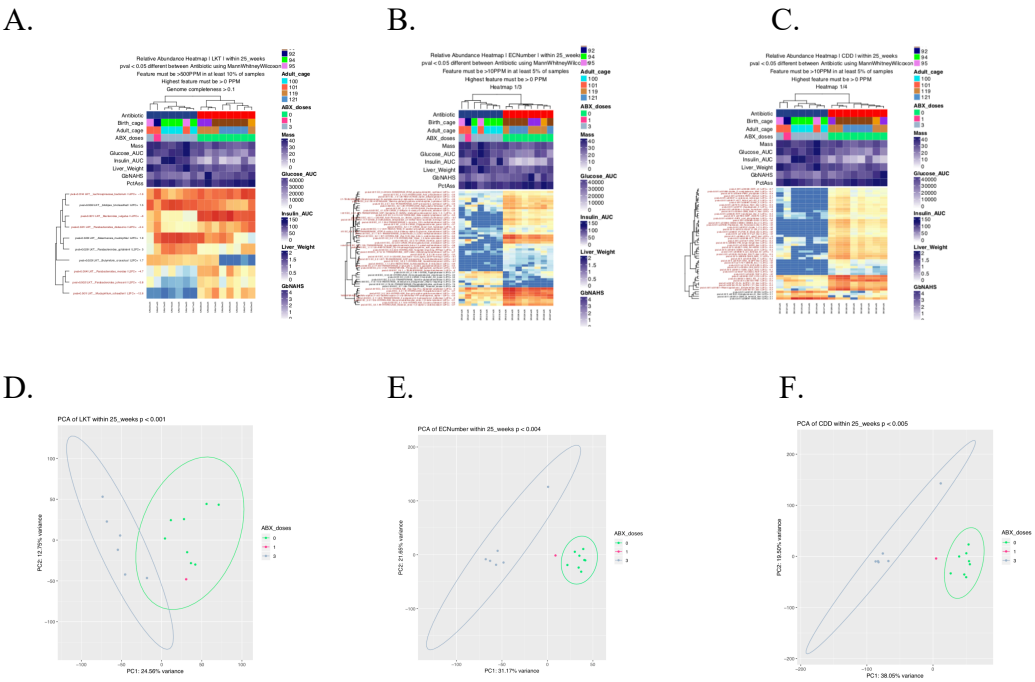


Figure 4. Stool from female offspring of dams treated with IP antibiotics prior to delivery and then placed on a western diet at weaning was shotgun sequenced after 25 weeks on the diet. Heatmap of the significant differences in the relative abundance of bacteria by last known taxa (A). Heatmap (1st of 3) of significant genes encoding for enzyme differences (B). Heatmap (1st of 3) of significant genes encoding for conserved domain protein differences (C). Principal Coordinate Analysis of differences in genes corresponding to differences in last known taxa (D), genes encoding enzymes (E), and genes encoding conserved domain proteins (F) by the number of doses of IP antibiotics that the offspring's dam received prior to delivery. Values are considered significant when the p value or adjusted p value in multiple comparisons is < 0.05. Statistical differences are determined by Mann Whitney. Wilcoxon, with fold changes >1.5 log 2-fold change.

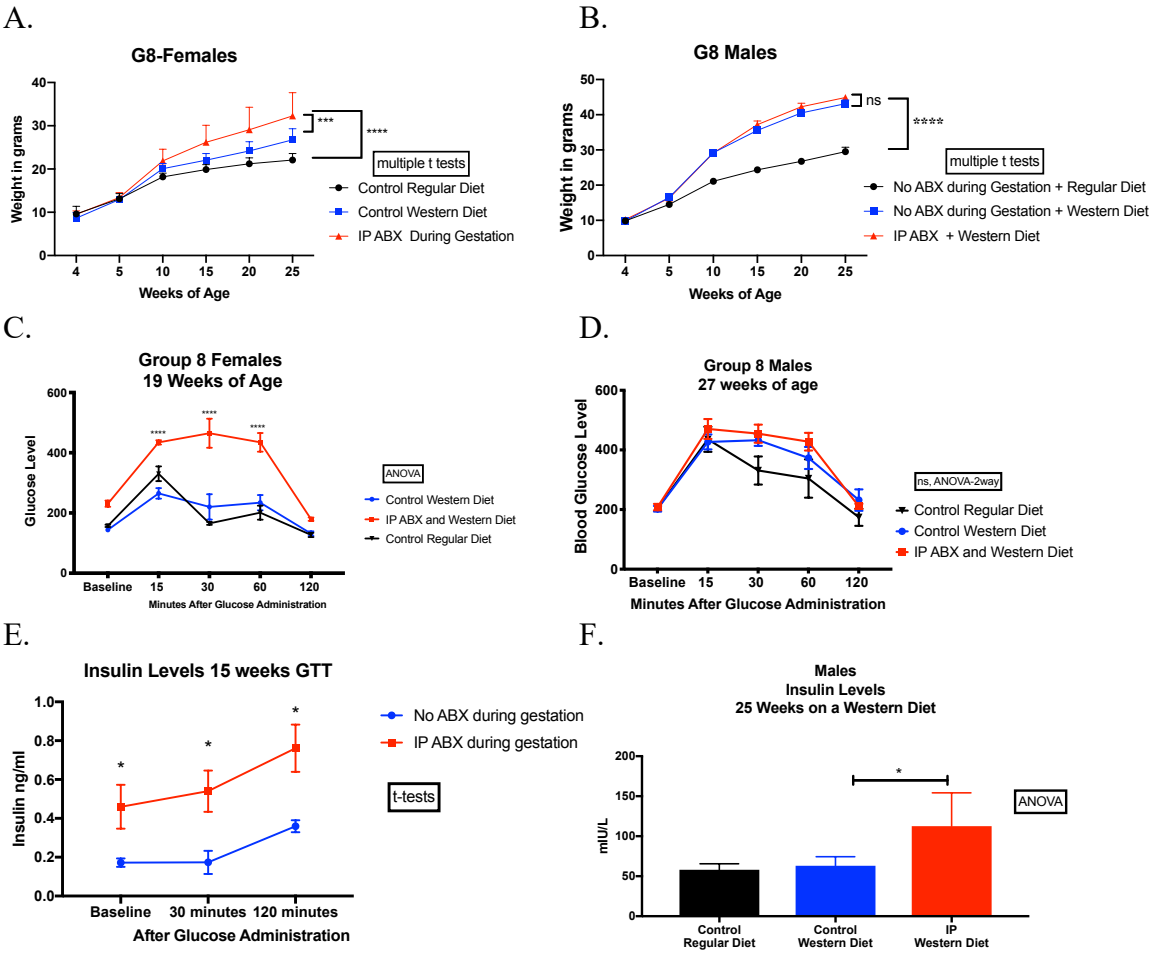


Figure 5. Female offspring of dams that received intraperitoneal (IP) antibiotics before delivery were significantly heavier on the western diet than female offspring whose mothers did not receive IP antibiotics before delivery (A). All males on the western diet were significantly heavier than those on regular chow, with no significant increases based on intrapartum antibiotic exposure (B). Glucose levels were elevated in response to the glucose tolerance test in female offspring whose mothers received antenatal antibiotics (C.) All males on the western diet had elevated blood glucoses during the glucose tolerance test. Those whose dams received antenatal antibiotics trended higher but were not significantly different than controls on the western diet (D). Female offspring exposed to antenatal antibiotics had elevated insulin levels during the glucose tolerance test (E). Male offspring exposed to antenatal antibiotics had elevated insulin levels at baseline (F). Graphs and statistical analysis generated using Graphpad PRISM 8.

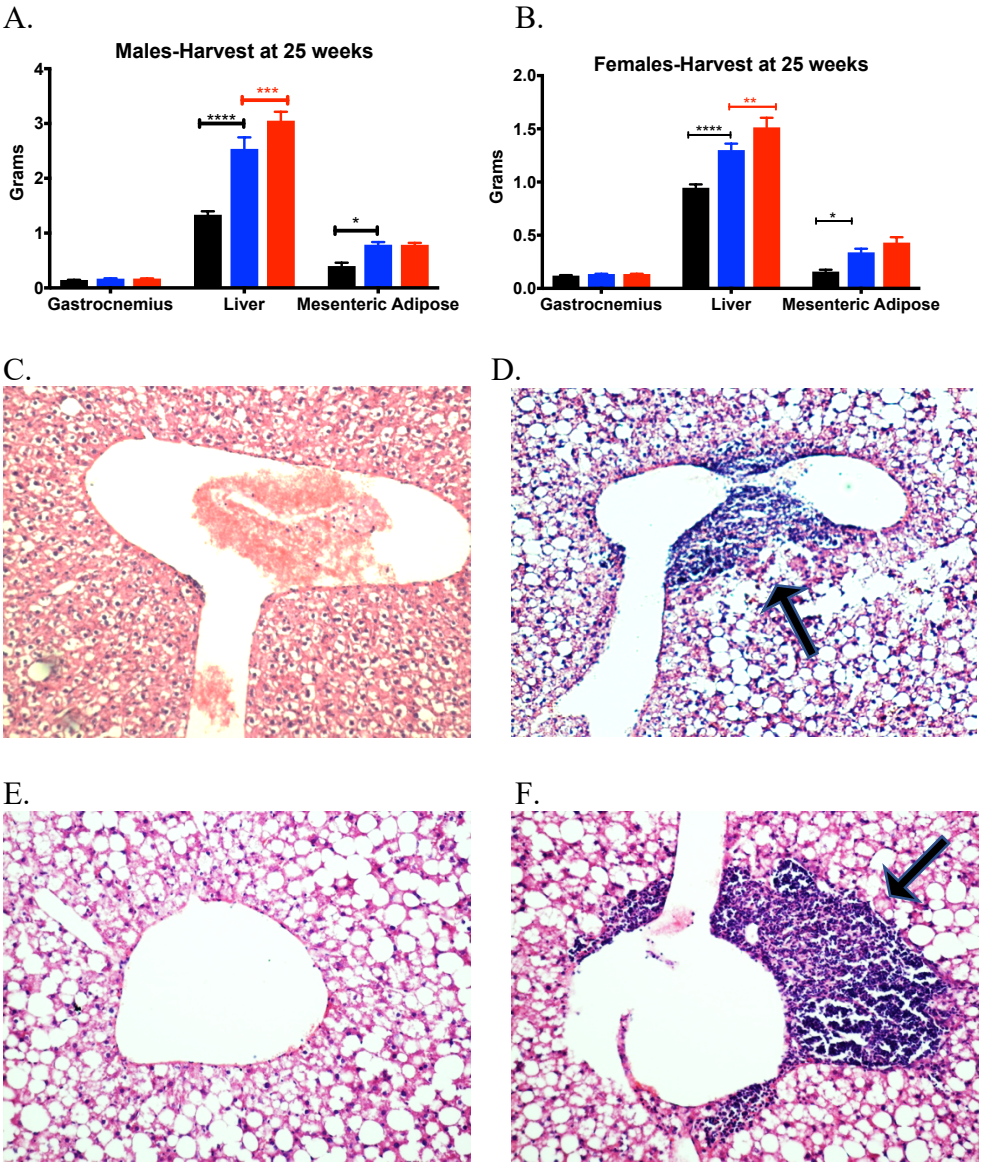
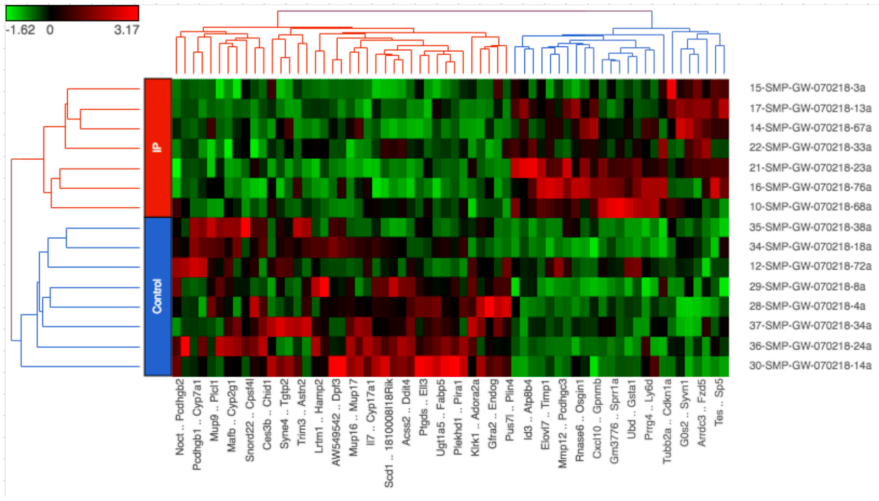


Figure 6. C57Bl/6 mice were 29 weeks old and were weaned onto a western diet at 4 weeks of age. Offspring of dams that received antenatal antibiotics were euthanized at 29 weeks. Male (A) and female (B) livers weighed more if their dams were exposed to 3 doses of intraperitoneal antibiotics prior to delivery. Liver segments were stained with Hematoxylin and Eosin and photos were taken at 10x magnification. Female adult offspring of a dam that received no antibiotics prior to delivery (C). Female adult offspring of a dam that received IP antibiotics (3 doses) prior to delivery (D). Male adult offspring of a control dam (E). Male adult offspring of a dam that received IP antibiotics (3 doses) prior to delivery (F). All photos lightened for easier viewing. Slides were generated from preserved liver tissues and stained by Histoserve. Arrows pointing to areas of focal inflammation.

A.



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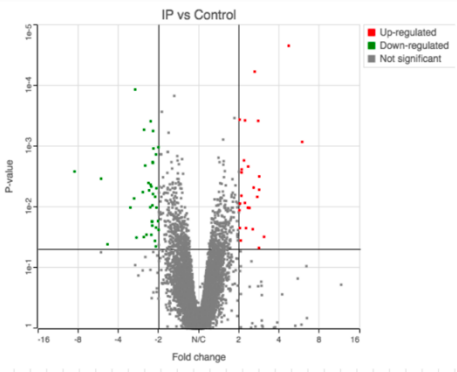

C.

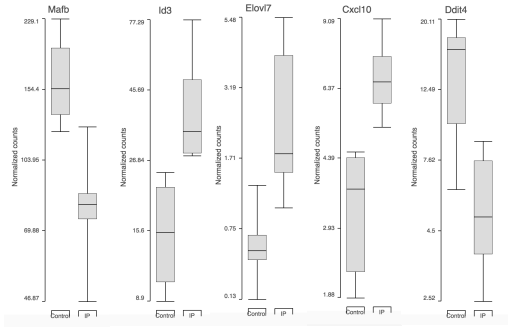

Figure 7. After 25 weeks on the western diet, adult female offspring whose dams did or did not receive IP antibiotics prior to delivery were euthanized. Liver RNA was sequenced using RNAseq and revealed transcriptional differences. Heatmap of liver RNA expression differences in female mice that received IP antibiotics $p<0.05$, log fold change >2 (A). Volcano plot of genes that were upregulated or down regulated in female offspring of mice that received antenatal antibiotics (B). Top 5 differentially expressed genes between IP and no antibiotic females on the western diet ($p<0.001$, log fold change >2, FDR <0.15) (C). Visualizations constructed in PARTEK flow.

A. B.

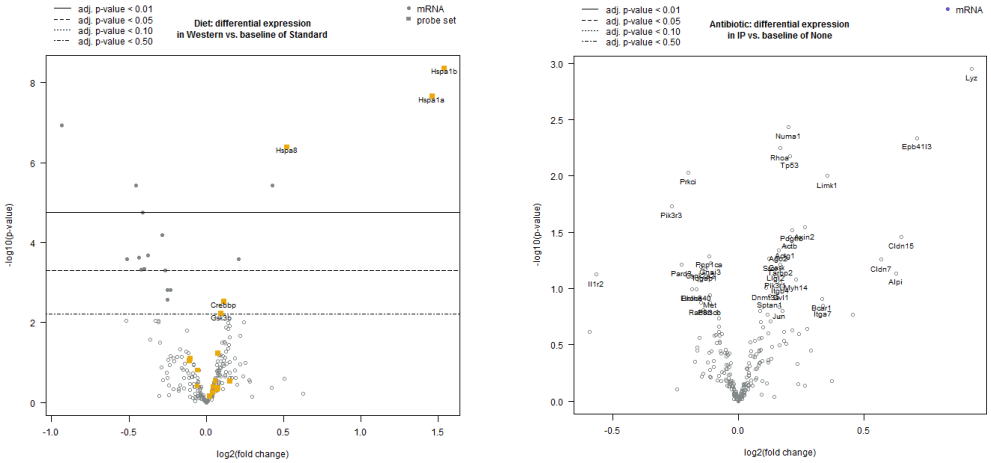

Figure 8. After 25 weeks on the western diet, adult female offspring whose dams did or did not receive IP antibiotics prior to delivery were euthanized. Intestinal RNA was sequenced using RNAseq and revealed transcriptional differences. Volcano plot of genes that were upregulated or down regulated in male and female offspring of mice that received the western vs. standard chow diet (A). Volcano plot of genes that were differentially expressed when comparing mice whose dams received IP antibiotics to those that received no antibiotics.

Conclusion

Experiments with pregnant mice demonstrate that their microbiota is altered by both oral and injected antibiotics, even when administered in limited doses prior to delivery. Exposed dams have reduced bacterial richness, and depletion of genera in the Bacteroidetes phylum and many unclassified bacteria at the time of delivery. Bacteria with antibiotic resistance have a competitive advantage when colonizing the antibiotic-perturbed maternal microbiota. These changes to the maternal microbiota are transmitted to offspring from birth and lead to altered colonization and decreased bacterial richness throughout the time periods tested in this study, which correspond to middle age in mice.

Reduced bacterial richness manifests itself in decreased richness in genetic pathways associated with functional proteins such as enzymes in the colonizing bacterial population, decreasing the resilience of the resident microbial population to stress and further perturbations, though it did not significantly alter the expression of genes in the host gastrointestinal tract. In this study, offspring exposed to stress-inducing stimuli, such as the western diet, had increased blood insulin levels and low-grade liver inflammation if their dams were exposed to prophylactic doses of antibiotics prior to delivery. Female antibiotic-exposed offspring additionally had decreased glucose tolerance and increased obesity on the western diet. All males on the western diet were glucose intolerant and obese, though maternal antibiotic exposure did lead to non-significant trends in increased weight gain and glucose intolerance.

Shotgun sequencing revealed that fecal bacterial populations in obese female offspring of IAP exposed dams had bacterial populations with increased genes for

inflammatory enzymes such as GGT, which may contribute to bacterial translocation and inflammation. Genes for inflammatory chemokines such as *CXCL10*, and inflammatory markers such as *Mafb* were upregulated in the livers of female obese IAP-exposed offspring, and these livers did exhibit increased steatosis and low grade inflammation and focal immune infiltrates, though the mechanism for these changes remains to be elucidated.

Although murine studies cannot be directly applied to humans, they do reveal important relationships between colonizing bacteria and host metabolism and immune response. Changes in bacterial colonizing species at critical developmental periods may alter host metabolic and immune programming. Reductions in the full spectrum of colonizing bacteria may also reduce environmental resiliency allowing invasion by species less beneficial or even pathogenic. The mechanisms by which bacteria influence the host are being uncovered by researchers daily in this emerging field. It is likely that host genetics and environmental stressors both play a role in healthy host-microbiota interactions.

As nurses are key intermediaries between patients and their environments, it is important that they understand how the microbiota affects a human host during pregnancy, at delivery, during development, and throughout the lifespan. As nurse practitioners are prescribers of medications and diets and nurse midwives oversee mothers throughout gestation and delivery, it is crucial to understand the impact that occurs not only directly to the patient, but also indirectly to the patient via changes to his microbiota. Understanding host-microbial interactions may lead to new methods of

maintaining health by stabilizing beneficial microbial populations, or to new therapeutics by supporting or emulating important microbial products that alter human metabolism and immunity.