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The Effect of Maternal Diet on the Establishment and Development of the Offspring's Gut Microbiome Studied in a Healthy Mouse Model

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THE EFFECT OF MATERNAL DIET ON THE ESTABLISHMENT AND DEVELOPMENT OF THE OFFSPRING'S GUT MICROBIOME STUDIED IN A HEALTHY MOUSE MODEL

BY

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TABLE OF CONTENTS

Abstract

The mammalian gastrointestinal tract has a diverse community of microbial species, collectively referred to as the normal flora, that influence the health of the host. Early colonization of this location is essential for the development of a newborn as the presence of these microbes influence the establishment of the immune system, metabolism, and allergy development. Recent research suggests that the fetal gut is colonized in utero and can be influenced by maternal factors, such as diet. Therefore, the objective of this study was to determine if the diet of the mother alters the establishment and development of the offspring's gut microbiome, reflected in a healthy mouse model. The ketogenic diet, characterized by high-fat and low-carbohydrates, and a standard pellet diet served as the experimental treatments. The fecal content of the offspring, born to mothers on each respective diet throughout pregnancy, was analyzed over a 32-week period to track the presence of particular bacterial species throughout development. This project identifies seven species as potential "core gut bacteria" that were used as markers of good health in monitoring the progression of the gut microbiome and changes in response to the ketogenic diet. Species-specific PCR primers were designed for each bacterium based on a unique target gene; however, time constraints prevented the resulting products from being analyzed. In the future, the amplified products from each species-specific PCR setup should be verified by gel electrophoresis and analyzed to determine the relative abundance of the seven bacteria.

Introduction

The mammalian gastrointestinal tract is home to a diverse range of bacterial species, often exhibiting mutualistic and/or commensal relationships with the host. The microorganisms that inhabit this location, as well as the skin and mucous membranes, are collectively referred to as the normal flora. The presence of microorganisms in the mammalian gut is specifically important as early colonization contributes to the development of a newborn, including the functioning of the immune system, metabolism, and health later in life (Walker et al., 2017). Once established, a healthy gut provides a range of benefits, including the ability to metabolize complex polysaccharides, detoxify harmful products, and to protect against pathogens (Tanaka & Nakayama, 2017).

However, the microbial composition of a stable gut is not fixed, but rather varies based on the health status and life stage of the organism. Factors such as genotype, age, environment, antibiotic exposure, and diet can alter the composition of the gut microbiome (Wang et al., 2019). In particular, the ketogenic diet (KD), a high-fat and low-carbohydrate regimen, has been associated with alterations to the normal gastrointestinal flora. The effects of the ketogenic diet have been explored in murine models, often linked to an increase in beneficial gut bacteria (Ellerbroek, 2018). Recent studies have suggested that the maternal diet can affect the colonization of the gut in pups (Laukens et al., 2016). Therefore, the purpose of this study was to determine if the maternal diet during gestation altered the establishment and development of the offspring's gut microbiome, specifically analyzing the ketogenic diet.

Vertical Transmission of Commensal Bacteria

It is traditionally believed that bacterial colonization of the offspring's intestinal tract begins during the birthing process, due to interactions with the mother's vaginal and fecal flora (Tanaka & Nakayama, 2017). Then, as the fetus interacts with the environment and consumes the mother's milk, high in microbes and immunoglobulins, further colonization occurs. This dogma, known as the sterile womb hypothesis and also termed the sterile womb paradigm, dates back to as early as 1885 when Theoder Escherich studied the meconium, or first stool sample, from a human fetus (Perez-Muñoz et al., 2017; Stinson et al., 2019). Escherich is credited as one of the first to study the fetal meconium which he described as sterile despite limitations in microscopy and microbial techniques (Perez-Muñoz et al., 2017). His findings created an avenue to study microorganisms present in fecal matter and were later echoed by many independent studies. Hall & O'Toole (1934) studied meconium samples from 50 healthy babies by analyzing sterile diapers. Similar to Escherich, 62% of the fecal samples were determined to be negative for bacteria by aerobic and anaerobic cultures, suggesting that the fetus develops within a sterile environment.

However, Jiménez et al. (2008) challenged this long-standing hypothesis with a study identifying bacteria in the meconium of healthy neonates obtained by caesarean section. The bacteria identified in the sample were predominantly of the *Enterococcus* and *Staphylococcus* genera which reflected the mother's gastrointestinal composition. Another experiment, conducted by the same research group, orally administered a genetically labeled strain of *E. faecium* to pregnant mice. The results revealed that bacteria could be isolated from the umbilical cord blood of neonates and was detected in the offspring whether born from a vaginal or C-section birth (Jiménez et al., 2005). This work indicates that bacteria can

be transferred from the oral cavity to the uterus via the bloodstream and suggests that there is vertical transmission of commensal bacteria prior to birth and independent of delivery mode (Walker et al., 2017). These findings have led to the in utero colonization hypothesis, which posits that the fetal microbiome is acquired before birth.

Further analysis has revealed the presence of bacteria in fetal membranes and amniotic fluid. Until recently, the presence of bacteria at these locations in humans was interpreted as evidence of infection or cause for pre-term delivery (Walker et al., 2017). However, the murine study by Jiménez et al. (2008) showed that bacteria are present during healthy pregnancies as well. It was determined that the maternal digestive tract was the origin of the bacteria found in the amniotic fluid (Jiménez et al., 2008). Since the fetus is constantly surrounded by and swallowing the amniotic fluid, the gastrointestinal tract is colonized, resulting in the presence of bacteria in the first fecal sample. Additionally, Gosalbes et al. (2013) isolated bacteria from the fetal meconium and classified the microbiota as members of the phylum *Firmicutes,* which contains many of the genera referenced later in this study. The findings from Jiménez et al. (2008) and Tanaka & Nakayama (2017) on the vertical transmission of bacteria in utero, serve as the rationale for studying the effect of the maternal diet in this research project.

Significance of Ketogenic Diet

The ketogenic diet (KD) has grown in popularity as a treatment for gastrointestinal diseases, obesity, and neurological disorders. As a low carbohydrate diet, the body resorts to using ketone bodies as the primary energy source (Ellerbroek, 2018). Microbiology research often utilizes the ketogenic diet as studies have shown it alters the gut microbiome by decreasing the overall microbial diversity due to the low polysaccharide content that the

bacteria rely on as fuel (Paoli et al., 2019). However, an increase in the abundance of beneficial species is often observed as they out-compete other species. This results in the reduction of many pro-inflammatory bacterial species which do not have a high survival rate when relying on ketone bodies as an energy source. Therefore, the reduction in inflammation leads researchers to study the effects of KD in irritable bowel disease (IBD), Chron's disease, and other gastrointestinal diseases (Comito et al., 2014). These KD studies indicate that diet plays a key role in influencing the overall composition and abundance of the gastrointestinal flora.

Recent findings have identified core members of the gut microbiome that directly correlate to the health and development of the host. Such species will be outlined in the coming sections and argued as essential bacteria to the healthy functioning of the murine gut. Therefore, the absence of such "core member" species can lead to dysbiosis and has been associated with inflammatory bowel disease, irritable bowel syndrome, and obesity (Kverka et al., 2011; Wang et al., 2019).

Core Members of the Gut Microbiome

The purpose of identifying "core members" of the gut microbiome in this research project is to compare how the presence of these bacteria change throughout development in the KD gestational offspring.

Laura Nee (2017) documented bacterial markers of good health in a thesis study at Trinity College by observing a strain of mice (BTBR T + tf/J) that display autistic symptoms both on and off a ketogenic diet. Nee identified *Clostridium disporicum* and *C. leptum* as core intestinal bacteria in both KD and non-KD mice. As a highly varied, anaerobic, and typically spore-forming genus, *Clostridium* species tend to be associated with many states of

health. In particular, *C. leptum*, which belongs to the *Clostridium* cluster IV, is essential in early life exposure as low gastrointestinal levels are linked to asthma and allergy development (Huang et al., 2015; Guo et al., 2020). Additionally, as an important carbohydrate-fermenting bacterium, reductions in *C. leptum* can also contribute to IBD, resulting in reduced short chain fatty acid production that is characteristic of the disease (Kabeerdoss et al., 2013). These two *Clostridium* species dominate the gut bacteria in mice and function in maintaining a healthy microbial community structure and the maturation of the immune system (Huang et al., 2015), proving them to be suitable markers for this study.

As part of the *Clostridium* cluster IV, the bacterium *Facecalibacterium prausnitzii* has been identified as a beneficial gut microbe, accounting for about 8% of the total mammalian colonic microbiota (Heinken et al., 2014; Guo et al., 2020). This bacterium is important for its production of butyrate, which serves as an energy source for other intestinal bacteria and has anti-inflammatory properties (Kabeerdoss et al., 2013). Low presence of this bacterium has been directly linked to the development of Crohn's disease and ulcerative colitis, further showing it to be an essential gut microbe (Loh & Blaut, 2012; Martín et al., 2017). As the *Clostridium* cluster IV bacteria utilize carbohydrates for fermentation pathways, the ketogenic diet, as a low-carb diet, is likely to impact the relative abundance of these species.

Nee (2017) also showed that *Bifidobacterium longum* and *Ruminococcus albus* were commonly present in the mammalian gastrointestinal tract. *Bifidobacteria* have been noted as early colonizers of the gut as the low oxygen levels provide a suitable environment for these anaerobes. This group of bacteria establishes the gastrointestinal lining in the early stages of life by producing mucins to form a barrier between the epithelial cells and the lumen

(Laukens et al., 2016). Research has shown that *B. longum* is abundantly present in the gut as it produces exopolysaccharides that can be used as fermentable substrates by neighboring bacteria (Nee, 2017). Similarly, *R. albus* contributes to the gastrointestinal microbial community by breaking down fiber present in the diet. Christopherson et al. (2014) defines *Ruminococcus* species as part of the core gut microbiome as the bacteria are found in 90% of healthy samples. Therefore, *B. longum* and *R. albus* are species of interest in this study as they have been reported as early gut colonizers, which can be used to compare the microbiota present in control diet versus KD gestational offspring.

Wang et al. (2019) analyzed the core gut bacteria of healthy mice by assessing fecal samples at time points similar to this research project. They defined "core gut bacteria" as present in at least 50% of the healthy mice samples regardless of age, sex, and strain, and reported that the genera *Clostridium, Ruminococcus, Facecalibacterium, Bifidobacterium, and Bacteroides,* accounted for 50-100% of the gut microbiota of healthy individuals. However, *Anaerostipes butyraticus* was present in the gut of every healthy mouse. This bacterium contributes to the host's health by protecting against colonic diseases. It consumes acetate to produce butyrate which is an important substrate for the proliferation of regulatory T cells and the reduction of gut inflammation (Eeckhaut et al., 2010). Similarly, *Parabacteroides distasonis* was present in 99% of samples causing Wang et al. (2019) to classify it as a core member of the gut microbiome. The presence of *P. distasonis* reduces the possibility of ulcerative colitis and IBD (Kverka et al., 2011). Additionally, through the activation of intestinal gluconeogenesis, *P. distasonis* alleviates obesity and related dysfunctions in mice by the production of succinate (Wang et al., 2019). Among the species

Nee (2017) identified, *A. butyraticus* and *P. distasonis* are considered essential gut bacteria in this study.

The goal of this research project was to determine if the maternal diet during pregnancy influences the development of the offspring's gut microbiome. Specifically analyzing the ketogenic diet, the use of Polymerase Chain Reaction (PCR) and gel electrophoresis provides insight on how the offspring's gut flora developed and identifies the specific bacterial species present in the fecal samples. The aforementioned bacteria act as the markers for healthy gut development and the relative abundance of species shows the impact the ketogenic diet has when fed during gestation.

Materials and Methods

Sample Selection

Fecal samples (180-220 mg) were collected from the offspring of healthy C57 Black mice divided into two parent groups. Group A breeder mice (n= 2) were fed the standard pellet diet (SD) throughout gestation while Group ZZ breeders (n= 2) were sustained throughout gestation on the ketogenic diet, resulting in normal and KD litters. On average, a litter resulting from either breeder groups contained 5-7 pups. The offspring nursed on the breeder females from their parent group; however, all pups were transitioned to the standard pellet diet at the time of weaning around 21 days post-birth. They stayed on the standard pellet diet throughout the remainder of the study, ensuring that the only variable in question was the maternal diet during gestation. All data were pooled for each group of offspring to determine the relative abundance of bacteria present. Therefore, individual mice were not labelled.

Nutritional Profile of Standard Diet

The standard pellet diet, consisting of 23% crude protein, 4.5% crude fat, 6% crude fiber, and 58.5% crude carbohydrates, served as the control in this project. The standard laboratory rodent diet (5001) was manufactured by LabDiet® (St. Louis, MO). Both groups of breeder mice were fed this diet prior to this project. However, Group ZZ breeder mice were transitioned to the ketogenic diet in the weeks prior to mating. It is reported that a change in diet alters the composition of the gut microbiome within 24 hours of initiation, therefore maternal diet prior to this experiment was not a major concern (Singh et al., 2017).

Nutritional Profile of Ketogenic Diet

In comparison, the typical ketogenic diet consists of 80% of the daily energy intake from fat, 15% from protein, and 5% from carbohydrates (Ellerbroek, 2018). However, the relatively low protein content of this diet is not sustainable for pregnant and nursing mice, often contributing to organ dysfunction or fatalities in research studies (Sussman et al., 2013). Therefore, a modified ketogenic diet suitable for rodents was used, consisting of 18% protein, 69% fat, 5% fiber, and 2.7% carbohydrates. The customized ketogenic diet (F5140) was purchased from Bio-Serv (Frenchtown, NJ). Additionally, all experimental protocols for working with mice on the ketogenic diet were approved by the Trinity College Institutional Animal Care and Use Committee (IACUC).

Collection Method & Time Points

Post-birth, female mice groom their offspring to stimulate urination and the passage of the meconium. The first stool sample is an accurate depiction of the bacterial content transferred from the gastrointestinal tract of the mother (Tanaka & Nakayama, 2017). Previous researchers have sacrificed newborn mice to obtain this sample. However, in an

attempt to obtain the meconium during a longitudinal study, a moistened cotton swab was used to mimic the licking action of the mother. This method produced varying success rates and the fecal content collected was not enough to quantify the bacterial DNA.

Therefore, sample collection began when the pups could defecate on their own, usually occurring around 3-4 weeks of age when the mice are weaned. The timeline for collection was at 4, 8, 12, 16, 24, and 32 weeks of age from the offspring of groups ZZ and A (Table 1). However, due to a delay in setting up breeder cages as a result of the COVID-19 pandemic, the 32-week sample for Group A (Non-KD gestational mice) could not be obtained as it fell outside of the projected time frame for this study.

As mice mature, their gut community becomes more stable (Schloss et al., 2012). This change is typically observed around week 12, signifying adulthood as the intestinal microbiota reach a homeostatic state (Laukens et al., 2016). The purpose of collecting fecal samples beyond this point was to determine how the relative abundance of species and overall composition of the gut microbiome changed throughout development. Therefore, the bacterial species present were identified at each given time point. However, the time interval between collection points increased closer to 32 weeks of age as little change in the gut composition was predicted.

Samples from the SD and KD gestational mice were taken by transferring them to a sterile cage for 5-10 minutes to obtain fresh pellets. The fecal samples were then labelled and stored in the freezer at -80 °C prior to processing.

Table 1. Fecal Sample Collection Dates and Date of Birth (DOB) for Ketogenic (ZZ) and Non-Ketogenic Diet (A) Gestational Litters

	Diet Group	
Sample	**ZZ (DOB, 8/3/20)**	**A (DOB, 10/16/20)**
4-week	9/1/20	11/13/20
8- week	10/5/20	12/11/20
12-week	11/2/20	1/8/21
16-week	11/30/20	2/5/21
24- week	1/25/21	4/2/21
32-week	3/22/21	*5/28/21

*Indicates sample will not be included in this project as the collection date falls outside of the accepted time frame

DNA Isolation & NanoDrop Analysis

The QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Inc., MD, USA) was used to isolate bacterial DNA from the samples following the "Isolation of DNA from Stool for Pathogen Detection" protocol. The resulting eluate from each sample was analyzed using a Thermo Scientific™ NanoDrop™ 1000 Spectrophotometer to quantify the amount of DNA present and determine the purity by recording the A260/280 values. For accurate absorbance readings, the NanoDrop was blanked with the elution buffer (Buffer ATE) prior to recording values for each sample.

Design of Species-Specific PCR Primers

To identify the bacterial species present in the fecal samples, primers for Polymerase Chain Reaction (PCR) were designed to target for unique properties of the bacteria. Nee (2017) designed primers for *B. longum, C. disporicum, C. leptum,* and *R. albus* using

sequences in the 16s rRNA gene that correspond to the specific bacterial species; these primers are utilized in this study. To run the polymerase chain reactions, Nee (2017) used two fluorescently labeled universal primers, 27F-5'-AGAGTTTGATCCTGGCTCAG-3' and 926R-5-'CCGTCAATTCMTTTRAGTTT-3', which generate an expected product of approximately 1000 base pairs. These universal primers bind to regions of the 16s rRNA gene that are highly conserved, therefore slight variation in the nucleotide sequence is indicative of a specific species. However, the full 16s rRNA gene has to be sequenced in order to determine the nucleotide changes which is not time efficient. Therefore, referencing Nee's work, I designed primers for *A. butyraticus, F. prausnitzii,* and *P. distasonis* by selecting for vital genes that contribute to the unique properties of the bacteria, rather than the universal 16s rRNA gene.

Unique genes for the species mentioned were discovered by searching the primary literature (Table 2). The butyryl-CoA: acetate-CoA transferase gene was selected as the target of interest for *A. butyraticus.* This gene carries out the final step in butyrate synthesis and was detected in eight strains of butyrate-producing bacteria, one of which was *A. butyraticus* (Eeckhaut et al., 2011). The choloylglycine hydrolase gene was the target for identifying *P. distasonis* in the isolated DNA samples. This essential gene contributes to primary bile acid metabolism which is a characteristic function of *P. distasonis* (Li et al., 2020). The gene used to generate primers for *F. prausnitzii* was the 16s rRNA gene. In searching the primary literature, Martín et al. (2017) designed *F. prausnitzii* specific forward and reverse primers, which were utilized in this research project.

However, for *A. butyraticus* and *P. distasonis*, once the unique genes were selected, the full nucleotide sequence was obtained using the National Center for Biotechnology

Information (NCBI) Nucleotide BLAST® tool. The full sequence was found by searching the gene name or FASTA sequence provided by the literature. The program selection was optimized for somewhat similar sequences (blastn) with the criteria of 100% Query Cover and Percent Identity. From the resulting hits, the sequences were compared to determine which best fit the criteria and matched the appropriate species. The gene sequence was then compared to related species in the same genus, using the NCBI Global Alignment tool, to determine the percent variability in the chosen sequence.

Table 2. Bacterial Species of Interest and Unique Target Genes

Bacteria	Specific Gene Targeted	Reference
A. butyraticus	butyryl-CoA: acetate CoA-transferase gene	Eeckhaut et al., 2011
B. longum	16s rRNA gene	Nee, 2017
C. disporicum	16s rRNA gene	Nee, 2017
C. leptum	16s rRNA gene	Nee, 2017
F. prausnitzii	16s rRNA gene	Martín et al., 2017
P. distasonis	choloylglycine hydrolase gene	Li et al., 2020
R. albus	16s rRNA gene	Nee, 2017

The NCBI Primer-BLAST® program was then used to generate potential species-specific PCR primers based on the selected nucleotide sequences. Final primers were selected using the criteria (Table 3) outlined in Nee's thesis (2017). The PCR primers were ordered through and produced by Invitrogen™ (Fisher Scientific, PA, USA).

Table 3. PCR Primer Design Criteria (Adapted from Nee, 2017)

Criterion	Value
Sequence Similarity to Bacteria in Same Genus	>10% difference
Length	15-25 base pairs
Hairpin	$\Delta G < 3.0$
Self-Dimer	$\Delta G < 5.0$
Heterodimer	$\Delta G < 6.0$
Melting Temperature	Within 2°C of the other
GC Content	40-65%
Presence of GC Clamp	Full or partial
PCR Product Length	500-1500 base pairs

Polymerase Chain Reaction & Gel Electrophoresis

Due to collection timeline setbacks outlined earlier, polymerase chain reactions could not be set up until all of the fecal samples were isolated for DNA. Campus alert levels in response to COVID-19 and semester breaks prolonged this process because I could not be on campus to work. Therefore, PCR setup was in the early stages when the deadline for this project approached. Due to this, only a few samples could be processed, however it was not enough to produce quantifiable results to draw conclusions.

PCR was performed using the "Protocol for One*Taq*® Quick-Load® 2X Master Mix with Standard Buffer (M0486)" manufactured by New England BioLabs®, Inc (Ipswich, MA). When using the universal primers, 27F and 926R, the polymerase chain reactions were placed in a thermocycler with settings for 95 °C initial denaturation, 50 °C primer annealing, and 72 °C extension for 30 cycles. The setup for the species-specific primers varied based on

the annealing temperature which is described in the coming sections. The resulting PCR products were run on a 2% agarose gel with 0.5x TBE Buffer to verify the banding patterns and fragment lengths of the bacterial DNA. The Quick-Load® 100 bp DNA Ladder (New England BioLabs, Ipswich, MA) was used as the standard for band comparison. Gels ran for about 1 hour at 170V and then were stained with ethidium bromide. The Trinity College Biology Department Kodak Gel Logic 100 Imaging System was used to document the gels. Given more time, this process would have been completed for all of the samples and refined to produce gels with legible banding patterns.

Results

Purity and Concentration of Isolated DNA Samples

NanoDrop values (Table 4) were obtained for a total of 11 samples from both ketogenic (ZZ) and standard diet (A) gestational mice. As mentioned previously, values for sample A-32 could not be gathered due to time constraints. However, for the remaining samples the purity, indicated by the A260/A280 value, and the DNA concentration (ng/μL) are displayed in Table 4. The code used to label each sample indicates the diet group for each offspring and the number of weeks at which collection occurred. Sample ZZ-16 contained the highest concentration of bacterial DNA with a value of 20.1 ng/μL. The lowest concentration was 1.7 ng/μL, belonging to sample A-24. In recognizing trends, ZZ samples, which correspond to KD gestational offspring, generally had a higher concentration of bacterial DNA (Table 4). The concentration of DNA was recorded to calculate the setup for PCR reactions, specifically the quantity of template DNA required for successful amplification.

The purity of the samples ranged from 260/280 values of 1.53- 2.48. A 260/280 ratio of approximately 1.8 is indicative of pure DNA. Samples collected from breeder A (standard

diet) mice, had 260/280 values close to 1.8, with the exception of A-16 (Table 4). Potential reasons for the deviation from this value are explored in the "Discussion" section.

Table 4. Nanodrop Analysis: Purity and Concentration of DNA Isolates from each sample taken from KD (ZZ) and Non-KD groups (A)

Sample	Weight (g)	A- 260 10 mm path	A-280 10 mm path	260/280	ng/µL
ZZ-4	0.086	0.148	0.084	1.76	7.4
ZZ-8	0.185	0.407	0.222	1.84	20.3
ZZ-12	0.960	0.073	0.035	2.06	3.6
ZZ-16	0.197	0.402	0.193	2.09	20.1
ZZ-24	0.184	0.183	0.117	1.56	9.1
ZZ-32	0.190	0.087	0.056	1.55	4.4
A-4	0.247	0.067	0.034	1.96	3.3
A-8	0.183	0.046	0.025	1.85	2.3
A-12	0.196	0.233	0.126	1.85	11.7
A-16	0.193	0.086	0.035	2.48	4.3
A-24	0.211	0.035	0.023	1.53	1.7

Designed Primer Pair Sequence

As described in the "Methods," there was not enough time to test the designed PCR primer pairs with the isolated DNA samples collected from group ZZ and A mice. However, Table 5 displays the designed primers that would have been used in the PCR setup for each unique species, consisting of the forward and reverse sequences and the expected product length (in base pairs). The smallest expected product length is 141 bp which corresponds to *F. prausnitzii* as determined by the primary literature (Table 5). The remaining expected

product lengths are close to 1000 bp which satisfies the primer design criteria outlined earlier.

Table 5. Species-Specific Primer Pair Sequence and Expected Product Length based on Target Gene

Species	Forward Primer (5'→3')	Reverse Primer (5'→3')	Product Length
A. butyraticus	GGAGGCATTGAAAGAAAATGGATCA	TCAGAGCTTCTGCACGTTCC	1009 bp
B. longum	GGCACTTTGTGTTGAGTG	CTCGACTGCGTGAAGG	837 bp
C. disporicum	CAGGGACGATAATGACGG	CCAGTTACGGGTAATTCAGG	514 bp
C. leptum	CCGCATAAGACCTCAGTACCGC	GGGATTTGCTTGCCTTCACAGGG	1042 bp
F. prausnitzii	CCATGAATTGCCTTCAAAACTGTT	GAGCCTCAGCGTCAGTTGGT	141 bp
P. distasonis	GTTTCAACCGCTTGTCGCTT	TATTTGCGATTGCCGGCTTG	1042 bp
R. albus	CGAGCGAAAGAGTGCTTGC	CGACTGCTTCCTCCTTGC	1332 bp

Based on the designed primers, the annealing temperature was calculated for each pair using the NEB Tm Calculator. The forward and reverse sequences (Table 5) and the melting temperature (Tm) at 50 mM Na^+ was used to produce the data in Table 6. Annealing temperatures ranged from 47-56 °C with the same denaturing and primer extension temperatures (Table 6).

Table 6. Polymerase Chain Reaction setup for Species-Specific Primer Pairs

Species	Denaturing Temp.	Annealing Temp.	Primer Extension Temp.
A. butyraticus	95 °C	51 °C	72 °C
B. longum	95 °C	47 °C	72 °C
C. disporicum	95 °C	48 °C	72 °C
C. leptum	95 °C	56 °C	72 °C
F. prausnitzii	95 °C	49 °C	72 °C
P. distasonis	95 °C	52 °C	72 °C
R. albus	95 °C	52 °C	72 °C

Although the PCR setup and gel electrophoresis confirmation could not be fully completed, Figure 1 displays the hypothesized timeline for the presence of each bacteria in the fecal samples collected throughout development in response to either maternal diet (KD or SD). The rationale supporting the timeline and the relative abundance of the bacterial species is outlined in the "Discussion" section.

Predicted Timeline Results

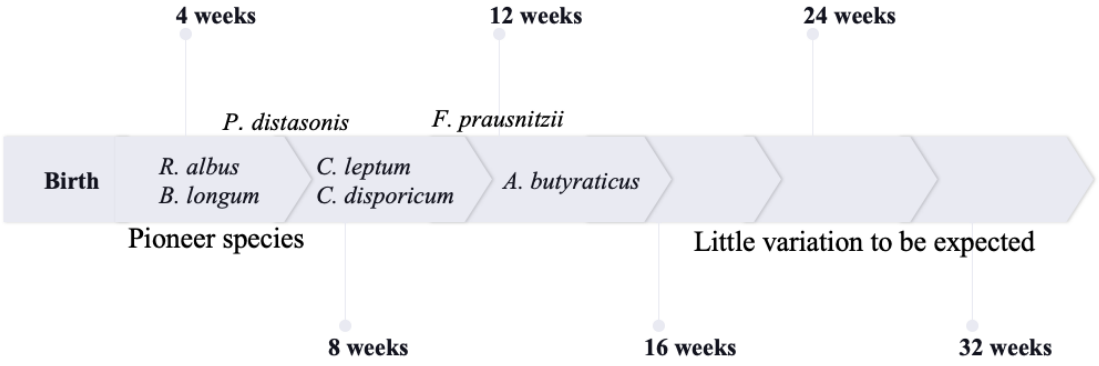

Figure 1. Predicted Presence of the Core Gut Species in KD gestational samples throughout the 32-week time period

Discussion

The goal of this study was to determine if maternal diet during gestation, either SD or KD, altered the establishment and development of the offspring's gut microbiome over a 32-week period. While the study could not be completed given time constraints due to the COVID-19 pandemic, the project was still successful in the design of species-specific PCR primers and the isolation of DNA from the fecal samples collected.

Purity and Concentration of Isolated DNA Samples

The NanoDrop values displayed that fecal samples from the offspring born to mothers on a ketogenic diet throughout pregnancy had a higher concentration of bacterial DNA

(Table 4). This finding is consistent with data reported in the primary literature as the ketogenic diet is associated with a decrease in the overall diversity of the gut microbiota, while increasing the abundance of beneficial species (Ellerbroek, 2018), such as the core gut bacteria previously defined. Additionally, there was a range of 260/280 values that fell above or below the universally accepted value of 1.8 for pure DNA. Ratios higher than 2.0, as seen for samples ZZ-12, ZZ-16, and A-16, possibly indicate RNA contamination which could result from reagents used in DNA extraction from the QIAamp® Fast DNA Stool Mini Kit (Matlock, n.d.). The higher ratio could also be due to contamination by environmental exposure or contact with the skin during sample processing (Matlock, n.d.). A ratio above 1.8 indicates that there could be an error in the blanking step as it suggests there is too much absorbance of the signal near the 280 nm wavelength (Matlock, n.d.). A 260/280 ratio lower than 1.8 is directly correlated to contamination from extraction buffers, in this case buffer ATE (Matlock, n.d.). However, deviations from the standard 1.8 value were generally very small so it is unlikely that they have a significant impact on the future acquisition of results.

Predicted Timeline Results

The first few fecal samples collected, either from group ZZ (KD gestational mice) or group A (SD gestational mice) are likely to contain the pioneer species *R. albus* and *B. longum.* As described in the section "Core Members of the Gut Microbiome" in the Introduction, these two bacteria are essential in the early stages of life for their role in establishing the gastrointestinal lining and the breakdown of fiber (Christopherson et al., 2014; Laukens et al., 2016), accounting for their placement in the timeline (Figure 1). It is hypothesized that mothers on either diet will have a relatively high abundance of either species that will be transferred to the offspring during gestation as they are "pioneer species".

Specifically, in a study analyzing changes to the colonic microbiome as a result of multiple sclerosis (MS), the ketogenic diet was shown to restore levels of *R. albus* to a high relative abundance (Swidsinski et al., 2017).

Similarly, Paoli et al. (2019) outlined a study that investigated the microbial differences in pediatric patients experiencing seizures that were fed a ketogenic diet. It was concluded that the relative abundance of *Ruminococcus*, like *R. albus,* and species of *Clostridia* (*C. leptum* and *C. disporicum*) increase in response to a ketogenic diet. These bacteria do not specifically rely on polysaccharides to conduct metabolic processes, therefore KD is not likely to negatively impact their presence. Therefore, as seen in Figure 1, *R. albus* and the two *Clostridium* species are likely to be present and at a high abundance in the early fecal samples (4 and 8 weeks) from KD gestational mice as they are essential in the early establishment of the gastrointestinal lining and the immune system (*C. leptum* and *C. disporicum)* (Guo et al., 2020).

A similar study analyzing the gut microbiota in response to KD for anti-seizure effects, found that species of *Parabacteroides* significantly increase in KD-fed mice (Olson et al., 2018). Therefore, considering the protective qualities associated with this bacterium, *P. distasonis* is hypothesized to be present in the early stages of development (Figure 1) and in high abundance in mothers on KD (Kverka et al., 2011). Murine models have also displayed that a high-fat diet, such as KD, results in a disproportionate increase in substrate-producing species, which *C. disporicum, C. leptum,* and *P. distasonis* are classified as (Singh et al., 2017).

Furthermore, Singh et al. (2017), reported that a high-saturated fat diet, such as the ketogenic diet, increases total counts of anaerobic microflora in the gut. Specifically, an

increase in the relative abundance of *Bifidobacterium* has been observed in mice (Singh et al., 2017). However, for human application, a ketogenic diet decreases the fecal abundance of *Bifidobacterium,* such as *B. longum,* for reasons not yet known (Singh et al., 2017). Likewise, both murine and human models have been shown to have a high relative proportion of *Faecalibacterium prausnitzii* in the gastrointestinal tract on a standard diet (Singh et al., 2017). However, it is hypothesized that *F. prausnitzii* will be present in a lower abundance in KD-fed mothers as the bacterium requires carbohydrates to produce butyrate by fermentation. This bacterium is essential in producing butyrate as it is used as an energy source for neighboring bacteria and has protective, anti-inflammatory properties (Kabeerdoss et al., 2013). Paoli et al. (2019) also found that a high-fat diet reduces the number of butyrate producers, specifically *F. prausnitzii*. Therefore, due to the low carbohydrate content of KD, this bacterium is not likely to be abundant in KD mothers, accounting for very little transfer to the offspring in utero. This bacterium is placed around the 12-week marker in the timeline in Figure 1. It is hypothesized that *F. prausnitzii* will appear in later samples after the offspring are transferred to their own independent diet (standard diet) and average carbohydrate levels are restored to support the presence of this bacterium.

By the same logic, *Anaerostipes butyraticus* is predicted to be present in the later fecal samples as it relies on the presence of carbohydrates to carry out fermentation. *A. butyraticus* specifically consumes acetate to produce butyrate which contributes to the proliferation of immune cells (Eeckhaut et al., 2010). It is hypothesized that like *F. prausnitzii, A. butyraticus* will not be present in high abundance in KD-fed mothers due to the low polysaccharide content.

In comparison, offspring born to mothers fed the standard diet during gestation are hypothesized to have some similarities to Figure 1, however the bacteria dependent on carbohydrates for fermentation (*F. prausnitzii, A. butyraticus,* and *P. distasonis*) are likely to be present in earlier samples. Between the 4-week and 8-week markers, it is predicted that all seven of the bacterial species will be present in the SD gestational offspring, however their relative abundance are likely to vary. For either diet group, it is hypothesized that little variation will occur in the composition of the gut microbiome past the 16-week collection time point (Figure 1) as the gut community has been reported to stabilize in this time frame (Laukens et al., 2016). Additionally, it is important to note that the studies referenced to support the timeline in Figure 1 do not specifically focus on the vertical transmission of bacteria from mother to offspring. As outlined in the experimental protocols, KD is fed directly to the offspring to produce the results rather than fed to the mothers. Therefore, this distinction could alter the predicted results as there are still many unknowns regarding in utero colonization.

Conclusions

Although definitive conclusions could not be drawn from this project as a product of the pandemic and condensed laboratory time, this study was still successful with the goal of learning more about how diet influences the mother-offspring pair by focusing in on these seven key species. Further research will construct a narrative of what particular species are present and the relative abundance at a given time throughout development to suggest if there is actually a benefit to the ketogenic diet in positively altering the gut microbiome. In a broader application, obtaining such results will contribute to the conversation on if the

ketogenic diet is beneficial for pregnant mothers and if their offspring are better equipped than those born on a standard diet for the potential in utero colonization of these core species.

Further Research

In the future, to confirm the accuracy of the designed PCR primers, isolated DNA from the seven species identified in this thesis as "core gut bacteria" should be obtained. Polymerase chain reactions should be run using the known bacterial species and the correlated primer pair to verify if the targeted gene is amplified. The resulting PCR products should be electrophoresed on a 2% agarose gel with a 100 bp ladder to compare the band size with the expected product size determined by the primer pair (Table 5) in order to confirm the presence of the aforementioned species in each fecal sample. This process should be completed for samples collected throughout the 32 weeks from KD gestational and SD gestational offspring to accept or reject the proposed timeline (Figure 1) of when these core gut species are likely to be present throughout development. Lastly, as another point of inquiry, quantitative polymerase chain reaction (qPCR) should be used to determine the relative abundance of the seven species in each of the samples, with the overall goal of determining how the ketogenic diet affects the gut microbiome in the mother-offspring pair.

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Literature Cited

Christopherson, M. R., Dawson, J. A., Stevenson, D. M., Cunningham, A. C., Bramhacharya, S., Weimer, P. J., Kendziorski, C., & Suen, G. (2014). Unique aspects of fiber degradation by the ruminal ethanologen Ruminococcus albus 7 revealed by physiological and transcriptomic analysis. *BMC Genomics*, *15*(1). https://doi.org/10.1186/1471-2164-15-1066

Comito, D., Cascio, A., & Romano, C. (2014). Microbiota biodiversity in inflammatory bowel disease. *Italian Journal of Pediatrics*, *40*, 32. https://doi.org/10.1186/1824-7288-40-32

Eeckhaut, V., Immerseel, F. V., Croubels, S., Baere, S. D., Haesebrouck, F., Ducatelle, R., Louis, P., & Vandamme, P. (2011). Butyrate production in phylogenetically diverse Firmicutes isolated from the chicken caecum. *Microbial Biotechnology*, *4*(4), 503–512. https://doi.org/10.1111/j.1751-7915.2010.00244.x

Ellerbroek, A. (2018). The Effect of Ketogenic Diets on the Gut Microbiota. *Journal of Exercise and Nutrition*, 1(5).

Guo, P., Zhang, K., Ma, X., & He, P. (2020). Clostridium species as probiotics: Potentials and challenges. *Journal of Animal Science and Biotechnology*, *11*(1), 24. https://doi.org/10.1186/s40104-019-0402-1

Hall, I. C., & O'Toole, E. (1934). Bacterial Flora of First Specimens of Meconium Passed by Fifty New-born Infants. *American Journal of Diseases of Children*, *47*(6), 1279–1285. https://doi.org/10.1001/archpedi.1934.01960130103007

Heinken, A., Khan, M. T., Paglia, G., Rodionov, D. A., Harmsen, H. J. M., & Thiele, I. (2014). Functional Metabolic Map of Faecalibacterium prausnitzii, a Beneficial Human

Gut Microbe. *Journal of Bacteriology*, *196*(18), 3289–3302. https://doi.org/10.1128/JB.01780-14

Huang, F., Qiao, H., Yin, J., Gao, Y., Ju, Y., & Li, Y. (2015). Early-Life Exposure to Clostridium leptum Causes Pulmonary Immunosuppression. *PLOS ONE*, *10*(11), e0141717. https://doi.org/10.1371/journal.pone.0141717

Jiménez, E., Fernández, L., Marín, M. L., Martín, R., Odriozola, J. M., Nueno-Palop, C., Narbad, A., Olivares, M., Xaus, J., & Rodríguez, J. M. (2005). Isolation of Commensal Bacteria from Umbilical Cord Blood of Healthy Neonates Born by Cesarean Section. *Current Microbiology*, *51*(4), 270–274. https://doi.org/10.1007/s00284-005-0020-3

Jiménez, E., Marín, M. L., Martín, R., Odriozola, J. M., Olivares, M., Xaus, J., Fernández, L., & Rodríguez, J. M. (2008). Is meconium from healthy newborns actually sterile? *Research in Microbiology*, *159*(3), 187–193. https://doi.org/10.1016/j.resmic.2007.12.007

Kabeerdoss, J., Sankaran, V., Pugazhendhi, S., & Ramakrishna, B. S. (2013). Clostridium leptum group bacteria abundance and diversity in the fecal microbiota of patients with inflammatory bowel disease: A case–control study in India. *BMC Gastroenterology*, *13*, 20. https://doi.org/10.1186/1471-230X-13-20

Kverka, M., Zakostelska, Z., Klimesova, K., Sokol, D., Hudcovic, T., Hrncir, T., Rossmann, P., Mrazek, J., Kopecny, J., Verdu, E. F., & Tlaskalova-Hogenova, H. (2011). Oral administration of Parabacteroides distasonis antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition. *Clinical & Experimental Immunology*, *163*(2), 250–259. https://doi.org/10.1111/j.1365-2249.2010.04286.x

Laukens, D., Brinkman, B. M., Raes, J., De Vos, M., & Vandenabeele, P. (2016). Heterogeneity of the gut microbiome in mice: Guidelines for optimizing experimental design. *FEMS Microbiology Reviews*, *40*(1), 117–132. https://doi.org/10.1093/femsre/fuv036

Li, M., Liu, S., Wang, M., Hu, H., Yin, J., Liu, C., & Huang, Y. (2020). Gut Microbiota Dysbiosis Associated with Bile Acid Metabolism in Neonatal Cholestasis Disease. *Scientific Reports*, *10*(1), 7686. https://doi.org/10.1038/s41598-020-64728-4

Loh, G., & Blaut, M. (2012). Role of commensal gut bacteria in inflammatory bowel diseases. *Gut Microbes*, *3*(6), 544–555. https://doi.org/10.4161/gmic.22156

Martín, R., Miquel, S., Benevides, L., Bridonneau, C., Robert, V., Hudault, S., Chain, F., Berteau, O., Azevedo, V., Chatel, J. M., Sokol, H., Bermúdez-Humarán, L. G., Thomas, M., & Langella, P. (2017). Functional Characterization of Novel Faecalibacterium prausnitzii Strains Isolated from Healthy Volunteers: A Step Forward in the Use of F. prausnitzii as a Next-Generation Probiotic. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.01226

Matlock, B. (n.d.). *Assessment of Nucleic Acid Purity*. 3.

Nee, Laura M. (2017). "An exploration of the gut bacteria in a mouse model of autism spectrum disorder on a ketogenic diet". Senior Theses, Trinity College, Hartford, CT. Trinity College Digital Repository, https://digitalrepository.trincoll.edu/theses/676

Olson, C. A., Vuong, H. E., Yano, J. M., Liang, Q. Y., Nusbaum, D. J., & Hsiao, E. Y. (2018). The gut microbiota mediates the anti-seizure effects of the ketogenic diet. *Cell*, *173*(7), 1728-1741.e13. https://doi.org/10.1016/j.cell.2018.04.027

Paoli, A., Mancin, L., Bianco, A., Thomas, E., Mota, J. F., & Piccini, F. (2019). Ketogenic Diet and Microbiota: Friends or Enemies? *Genes*, *10*(7). https://doi.org/10.3390/genes10070534

Perez-Muñoz, M. E., Arrieta, M.-C., Ramer-Tait, A. E., & Walter, J. (2017). A critical assessment of the "sterile womb" and "in utero colonization" hypotheses: Implications for research on the pioneer infant microbiome. *Microbiome*, *5*(1), 48. https://doi.org/10.1186/s40168-017-0268-4

Schloss, P. D., Schubert, A. M., Zackular, J. P., Iverson, K. D., Young, V. B., & Petrosino, J. F. (2012). Stabilization of the murine gut microbiome following weaning. *Gut Microbes*, *3*(4), 383–393. https://doi.org/10.4161/gmic.21008

Singh, R. K., Chang, H.-W., Yan, D., Lee, K. M., Ucmak, D., Wong, K., Abrouk, M., Farahnik, B., Nakamura, M., Zhu, T. H., Bhutani, T., & Liao, W. (2017). Influence of diet on the gut microbiome and implications for human health. *Journal of Translational Medicine*, *15*. https://doi.org/10.1186/s12967-017-1175-y

Stinson, L. F., Boyce, M. C., Payne, M. S., & Keelan, J. A. (2019). The Not-so-Sterile Womb: Evidence That the Human Fetus Is Exposed to Bacteria Prior to Birth. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.01124

Sussman, D., van Eede, M., Wong, M. D., Adamson, S. L., & Henkelman, M. (2013). Effects of a ketogenic diet during pregnancy on embryonic growth in the mouse. *BMC Pregnancy and Childbirth*, *13*(1), 109. https://doi.org/10.1186/1471-2393-13-109

Swidsinski, A., Dörffel, Y., Loening-Baucke, V., Gille, C., Göktas, Ö., Reißhauer, A., Neuhaus, J., Weylandt, K.-H., Guschin, A., & Bock, M. (2017). Reduced Mass and Diversity of the Colonic Microbiome in Patients with Multiple Sclerosis and Their

Improvement with Ketogenic Diet. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.01141

Tanaka, M., & Nakayama, J. (2017). Development of the gut microbiota in infancy and its impact on health in later life. *Allergology International*, *66*(4), 515–522. https://doi.org/10.1016/j.alit.2017.07.010

Walker, R. W., Clemente, J. C., Peter, I., & Loos, R. J. F. (2017). The prenatal gut microbiome: Are we colonized with bacteria in utero? *Pediatric Obesity*, *12*(S1), 3–17. https://doi.org/10.1111/ijpo.12217

Wang, J., Lang, T., Shen, J., Dai, J., Tian, L., & Wang, X. (2019). Core Gut Bacteria Analysis of Healthy Mice. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.00887

Wang, K., Liao, M., Zhou, N., Bao, L., Ma, K., Zheng, Z., Wang, Y., Liu, C., Wang, W., Wang, J., Liu, S.-J., & Liu, H. (2019). Parabacteroides distasonis Alleviates Obesity and Metabolic Dysfunctions via Production of Succinate and Secondary Bile Acids. *Cell Reports*, *26*(1), 222-235.e5. https://doi.org/10.1016/j.celrep.2018.12.028