Comparison of Gut Microbial Community in Infants and Toddlers with and without Phenylketonuria

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Abstract
Gut Microbial Community in Infants and Toddlers with and without Phenylketonuria (PKU)

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Background: Prebiotics are non-digestible food components that selectively stimulate the growth and/or activity of beneficial bacteria in the colon and contribute to improved human health. Infants and young children with inborn errors of metabolism (IEM), such as phenylketonuria (PKU) consume low amounts of dietary sources of prebiotics because of their comprehensive dietary restriction of phenylalanine-containing foods. It is unknown if the amount of prebiotics that patients with PKU consume provides adequate substrate to support a beneficial gut microbial community (GMC). The GMC is involved in many aspects of human health, including providing a barrier for colonization of pathogens, contributing to energy salvage and regulation, and modulating immune function.

Methods: We conducted an observational pilot study to characterize the GMC profiles of 6 patients with PKU and compare them to those of 13 age- and sex-matched controls. Study activities included (1) administration of a brief questionnaire to collect data about general health status, use of antibiotics, mode of infant delivery, gestational age at birth, birth weight, maternal diet and maternal pre- and probiotic use; (2) analysis of 3-day food records for nutrient and pre-biotic intake; and (3) assessment of GMC using bacterial DNA extracted from fecal samples.

Results: The questionnaire revealed no statistically significant differences between the two groups. The analysis of the 3-day food record revealed significant differences in protein, phenylalanine and dietary fiber intakes. There was an inadequate number of patients receiving breast milk or standard infant formula to compare prebiotic intake between the two groups. The assessment of GMC revealed bacteria were distributed across 5 phyla: Firmicutes (57%), Bacteroidetes (37%), Proteobacteria (2.3%), Verrucomicrobia (0.4%), and Actinobacteria (2.7%). There was no significant difference in the distribution of bacterial phyla between control and PKU subjects. There was no statistically significant difference in the Shannon diversity index, which is a measure of the evenness and richness of the GMC within a person, between controls and participants without PKU (p=0.16). Although not statistically significant, diversity tended to be lower in the children with PKU compared to controls.

Conclusion: This pilot study provided important initial insight into the GMC differences between patients with PKU and children receiving usual dietary care and generated important preliminary data needed for design of a larger, comprehensive study. Given that establishment of a healthy, diverse GMC may have implications for the long-term health of individuals, the ultimate goal of this study was to inform the development of optimized nutritional products that may improve the lives of individuals with PKU.
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Introduction

Formulas that have been supplemented with prebiotics can promote gut microbial communities (GMC) in formula-fed infants similar to those observed in breast-fed infants which may better support development of the immune system, resulting in clinically protective effects (1, 2). Because of these expected health benefits, oligosaccharide prebiotics are commonly added to commercially available standard infant formula products (3). Individuals with PKU require specialized metabolic formulas which do not currently contain prebiotic compounds, and this may negatively impact their GMC.

Human milk is widely recommended as the best source of nutrition for infants as it contains many bioactive components that support optimal growth and development of newborns, including gut microbial colonization, immune maturation and cognitive development (4). Emerging evidence shows that breastfeeding also has potential health benefits later in life, including decreased risk of several chronic diseases such as obesity, allergy, cardiovascular disease and diabetes (5, 6). Significant efforts have been made to characterize the beneficial bioactive compounds of breast milk in order to optimize the human milk substitutes that some infants may require (4). One group of beneficial bioactive compounds includes the nondigestible oligosaccharides, which are “the third most abundant fraction after lactose and lipids in human milk” (4). Human milk contains a substantial amount of oligosaccharides (20-23 g/L in colostrum and 14-18 g/L in mature milk) (1, 7). These nondigestible oligosaccharides have been called prebiotics because they selectively stimulate the growth and/or activity of a beneficial GMC (8).

Early life colonization of the GMC is crucial for healthy development; the human body hosts approximately 100 trillion bacteria, which is 10 times the number of cells of the human body, and there are approximately 500 to 1000 distinct bacterial species in the gut (4, 9, 10). The gene set of the human microbiome is 150 times larger than the human genome, with 3.3 million non-redundant genes (10). Host-microbiota symbiosis is crucial because the microbes contribute to many crucial biochemical processes, including degradation of food-derived and endogenous constituents, provision of
metabolites that serve as an energy source for enterocytes, detoxification of xenobiotic compounds, bioactivation of beneficial constituents such as polyphenols, basal stimulation of the immune system and epithelium, and prevention of colonization by, or excessive development of, pathogenic microorganisms (4). The beneficial GMC is characterized by greater total bacterial diversity and a greater prevalence of Lactobacillus and Bifidobacterium species (11).

The prebiotic compounds contained within human milk and the resulting impact that these prebiotic compounds have on microbial colonization have been extensively studied recently, and nutritional strategies designed to mimic the functionalities of human milk have been investigated (4). The addition of oligosaccharides to infant formulas to act as selective microbiota substrates is considered an efficient nutrition intervention that modulates the GMC in a way that is similar to that of human milk (4). The prebiotics added to infant formula are generally composed of a short-chain galacto-oligosaccharide (scGOS) and long-chain fructo-oligosaccharide (lcFOS) mixture (9:1); this mixture was designed to mimic the molecule size distribution of human milk oligosaccharides (12). Infants receiving a standard formula (without prebiotics) compared to a scGOS- and lcFOS-supplemented formula (0.8 g/100 mL) over a 6-week period showed a significant increase in total percentage of fecal Lactobacilli resulting in a GMC more similar to that of breast fed infants (13). Specifically, the Lactobacillus species distribution of the prebiotic formula-fed group was more comparable to breast-fed infants, with relatively high levels of L. Acidophilus, L. paracasei and L. casei (13). In a double-blind investigation, health formula-fed infants aged younger than 2 weeks were randomized to receive a scGOS+lcFOS-supplemented formula (0.8 g/100 mL) or a standard infant formula until the age of 12 weeks to examine the bifidogenicity of the oligosaccharide fortified formula. The infants who received the oligosaccharide-supplemented formula had higher counts of bifidobacteria in their feces than the infants who received the standard formula that did not contain the oligosaccharide blend, which is desirable since bifidobacteria are nonpathogenic and are "thought to protect infants from pathogenic intestinal microorganisms during a phase of insufficient immune response" (14). Other studies have also shown that infants receiving prebiotic-supplemented formula
have higher total amounts of fecal bifidobacteria with a diversity of Bifidobacterium species more similar to that of breast-fed infants than that of standard formula fed infants (15).

The impact of formulas supplemented with scGOS+lcFOS on microbial composition corresponds with changes in short chain fatty acid (SCFA) production, lactate and a decreased pH in the infant gut that resembles the fermentation pattern generated by human milk consumption (16). Prebiotic supplementation of infant formula not only results in fecal bacterial profiles reflective of a GMC that is more similar to that of breast-fed infants, but it has also been associated with many positive health outcomes including decreased risk of development of atopic dermatitis, improved immune function, decreased colic and decreased inflammation (1, 8, 17-19). A double-blind, randomized, placebo-controlled study of 259 term infants with parental history of atopic eczema, allergic rhinitis or asthma showed that infants receiving a hypoallergenic formula that was fortified with prebiotics (0.8 g scGOS+lcFOS/100 mL) were less likely to develop atopic dermatitis than the infants receiving the hypoallergenic formula without prebiotics (20). Supplementation of scGOS+lcFOS in infant formulas has been shown to induce a beneficial antibody profile, specifically demonstrating a significant reduction in plasma level of total IgE, IgG1, IgG2 and IgG3 (21). Healthy term infants with high risk of atopy who received a prebiotic-supplemented formula (0.8 g/100 mL scGOS+lcFOS) had fewer episodes of all types of infections, fewer upper respiratory tract infection episodes and fewer infections requiring antibiotic treatment than controls (22). In addition, the cumulative incidence of recurring infections during the 6-month study period was significantly lower in the scGOS+lcFOS group (22).

The health benefits and protective effects of early prebiotic consumption appear to extend beyond infancy (7). A two-year follow-up of 134 toddlers indicated that the cumulative incidence of atopic dermatitis, recurrent wheezing and allergic skin rashes was lower in the group that received the prebiotic supplemented formula (0.8g/100mL scGOS+lcFOS) during infancy than the infants who received a placebo-supplemented (0.8g/100mL maltodextrin) supplemented formula (7). In addition, the prebiotic-fed infants had fewer episodes of physician-diagnosed overall and upper respiratory tract infections, fewer reported fever episodes and fewer antibiotic prescriptions (7). The same research
group showed that early prebiotic supplementation reduced the incidence of allergic manifestations, including allergic rhinoconjunctivitis in the first 5 years of life for high-risk infants (23).

Individuals with phenylketonuria (PKU) are a nutritionally vulnerable population because the medical treatment for PKU is primarily achieved via dietary restrictions (24). PKU is a metabolic disorder characterized by a deficiency in the enzyme phenylalanine hydroxylase (PAH), which converts phenylalanine (PHE) to tyrosine (TYR) (25). Untreated, patients with PKU are at risk for cognitive impairment, as well as microcephaly, epilepsy, growth impairment, severe behavior problems, eczema, poor pigmentation, and psychiatric conditions such as depression, anxiety and phobias (25-27). The exact mechanism of neurological damage caused by untreated PKU is unknown; however, one hypothesis is that the impaired metabolism of PHE results in limited TYR, which is a precursor to dopamine, an essential neurotransmitter necessary for signal transmission between nerve cells (26). Additionally, recent neuroimaging studies have shown impaired myelination and white matter abnormalities in the brain of individuals with PKU, even those that were diagnosed early and treated continuously since diagnosis (28). Researchers have found an age-related decrement in the microstructural integrity of white matter of the corpus callosum in children with PKU (29). Although there may be some limitations of executive function in patients with PKU, it is possible to prevent the severe negative outcomes of significant cognitive impairment with treatment (30). As a result, PKU is a part of newborn screening programs in all 50 states and in many countries around the world (31).

Current treatment for patients with PKU includes implementation of a strict low-PHE diet to maintain blood PHE concentrations between 120 and 360 µmol/L (2-6 mg/dL), which prevents the development of severe neurological complications (25, 26). PHE is an essential amino acid, so some PHE must be provided for necessary protein synthesis to support tissue repair, growth and development during childhood and protein turnover during adulthood; however, the amount of PHE in the diet is closely monitored to maintain plasma PHE concentrations within recommended treatment ranges (24). Individuals with PKU require close monitoring of blood PHE concentrations and PHE
intake because PHE requirements can change with growth, body mass and age. Lifelong dietary treatment is recommended to maintain blood PHE concentrations within recommended range (31).

The prescribed low-PHE diet includes vitamin and mineral fortified synthetic PHE-free metabolic formula consumed multiple times per day (25, 26). PHE occurs naturally in all foods that contain protein, therefore individuals with PKU must use synthetic high protein, amino acid (AA) based, PHE-free formulas to achieve a nutritionally-complete diet that supports normal growth and development (24). Individuals with PKU eat a variety of low-protein foods in addition to the prescribed metabolic formula; however, even low-protein foods contain PHE, so foods must be carefully measured and portioned in order to maintain a PHE intake that will support goal blood PHE concentrations (24).

Typical food intake for individuals with PKU generally eliminates all sources of animal protein, legumes and nuts, as well as limits intake of regular bread, pasta, rice and some vegetables (24). The food intake of individuals with PKU consists mainly of specialty low-protein bread and pasta products made from starch to provide needed energy and to increase variety in addition to small amounts of fruits and vegetables (24).

The prescribed low-PHE nutrition therapy for individuals with PKU has limited natural sources of iron, calcium, omega-3 fatty acids, vitamin B12 and zinc. Although the PHE-free metabolic formulas provide adequate amounts of all the nutrients, nutrient deficiencies and the resulting sequelae may develop in individuals with PKU who do not drink the full amount of their formula prescription (32). Bone mineral density (BMD) status evaluations show severe osteopenia in children with PKU compared to controls, with bone loss being more prominent in patients over 8 years of age (33). PKU may affect the metabolism of other nutrients, possibly resulting in increased needs. For example, children with PKU have been observed to have iron deficiency, even with iron intakes greater than the DRI (34).

Individuals with PKU may be at risk for developing essential fatty acid deficiencies secondary to their restrictive diet. Concentrations of fatty acids, including total erythrocyte lipid of the sum of omega-3, omega-6, saturated and polyunsaturated fatty acids, among individuals with PKU have been shown to be lower than control subjects (35). Regardless, because of the importance of the long-chain
polyunsaturated fatty acids (PUFA) docosahexanoic acid (DHA) and arachidonic acid (AA) for neural
development, an increasing number of manufacturers are supplementing the metabolic formulas for
PKU with DHA and AA (36, 37).

Infants and young children with inborn errors of metabolism (IEM), such as PKU consume low
amounts of dietary food sources of prebiotics because of their comprehensive dietary restriction of
PHE-containing foods and use of synthetic metabolic formulas to meet the majority of energy needs to
support growth and development (32). Currently in the North American market, no synthetic PHE-free
metabolic infant formulas contain added prebiotic components. While infants with PKU may have some
prebiotics in their diet, either from small amounts of commercial formula or breast milk, the majority of
energy is supplied from specialized metabolic formulas, which are not currently fortified with prebiotics.
It is unknown if the small amounts of prebiotics that PKU patients consume provide adequate substrate
to support a beneficial GMC.

Administration of amino acid based diets induces spontaneous bacterial translocation to
mesenteric lymph nodes in animal models (38). Bacterial translocation is the passage of viable
bacteria from the gastrointestinal tract to other extraintestinal sites (39). One mechanism of promoting
bacterial translocation in animal models is the disruption of the ecologic GI equilibrium to allow
intestinal bacterial overgrowth (39). The formulas that individuals with PKU consume are amino acid
based; this may result in increased risk of a suboptimal GMC and may be another area of nutritional
vulnerability of this population.

Recently an exploratory study in the UK investigated the influence of adding prebiotic
oligosaccharides (scGOS/IcFOS 9:1 ratio) to metabolic formula for infants with PKU (40). The study,
an 8-week open-label, single-arm, pilot intervention in 9 infants between 4 weeks and 6 months of age
diagnosed with PKU, found that the prebiotic-supplemented metabolic formula was well tolerated (40).
Metabolic control and *bifidobacteria* levels were maintained, and stool pH was lowered (40). Although
no statistically significant change was observed in *bifidobacteria* or *lactobacilli-enterococci* from
baseline levels, there was a marked increase in *bifidobacteria* levels in two subjects who had the lowest
bifidobacteria concentrations at baseline (3.6% and 6.7% at baseline, increased by 54.6% at 27.9%) (40). Stool consistency as judged by the primary caregiver was also reportedly improved when infants received the prebiotic-supplemented formula (40). Currently in the UK, the metabolic formula PKU Anamix Infant (Nutricia) is supplemented with prebiotic fibers (0.8 g/100 mL scGOS+lcFOS) (41).

Objectives

The objectives of this observational pilot study were:

1. To compare the gut microbial profile of infants and toddlers with PKU receiving the majority of their nutrition from metabolic formula to sex- and age-matched controls with or without medium chain acyl-coA dehydrogenase (MCAD) deficiency, receiving a conventional infant diet.
2. To compare the prebiotic content of PKU infant and toddlers diets to those of controls via detailed diet analysis.

Hypothesis: The GMC differs between PKU and control participants; specifically Lactobacillus and Bifidobacterium species and total bacterial diversity were hypothesized to be lower in participants with PKU as compared to control participants. Further, we hypothesized that these differences would be most pronounced in participants with who are younger than 6 months of age and are exclusively formula (or formula and breast milk) fed.

Methods

1. Study population. Males and females (<3 years of age) were recruited from the Cristine M. Trahms Program for Phenylketonuria at the University of Washington. The Cristine M. Trahms Program for Phenylketonuria program follows all infants and children with PKU across the state of Washington. Monthly group clinic appointments are held in Seattle and are attended by patients and their families. Infants and children diagnosed with PAH deficiency and who required amino acid-modified formula to maintain blood PHE and TYR levels were included.

   Children less than 3 years old with medium chain acyl CoA dehydrogenase (MCAD)
deficiency who do not require specialized formulas, but receive similar routine clinical attention were recruited from the University of Washington Biochemical Genetics Program as controls. Additional controls who did not require specialized metabolic formulas were recruited from Kindering, a local not-for-profit early intervention program.

2. Screening and Recruitment. The initial study recruitment goal was to recruit 10 individuals with PKU and 10 individuals without PKU to serve as controls. Parents or legally acceptable representatives of eligible participants were contacted to inform them of the study. Recruitment occurred through mailed letters to the eligible participants who were followed at the Cristine M. Trahms Program for Phenylketonuria at the University of Washington and the University of Washington Biochemical Genetics clinic, phone calls and provision of written materials during routine clinic visits. An announcement about the study was placed in the Kindering monthly family newsletter from September 2013 until December 2013. In addition, the study staff attended selected early intervention classes at Kindering to meet with the parents of eligible children to answer any questions about the study and enroll children of parents who were interested participating. Expanded recruitment efforts were required due to initial low participation, and additional controls were obtained from the community. Signed informed consent on behalf of each participant was obtained.

4. Study Activities and Data Collection. Parents of participants (individuals with PKU and controls) completed a questionnaire about general health status both of mother and baby, antibiotic use, mode of infant delivery (vaginal or C-section), gestational age at birth, birth weight, and other health metrics (Appendix A). This questionnaire was adapted from one used previously by Penders et al who examined the contribution of a broad range of external influences on the gut microbiome in infants (42). This questionnaire was administered to identify variables that may impact the GMC. Anthropometric data were obtained by parent report via the questionnaire. The weight-for-age and length-for-age percentiles were obtained via use of online growth chart calculator which uses the WHO growth
standard charts for 0-24 months (http://peditools.org/growthwho/index.php), and for subjects over 24 months weight-for-age and height-for-age percentiles were calculated with the Baylor College of Medicine online calculator for age-based pediatric growth reference charts, which utilizes the CDC growth charts (https://www.bcm.edu/bodycomplab/Flashapps/bmiVAgeChartpage.html).

A patient chart review was conducted for the participants with PKU to obtain blood PHE levels as indicator of metabolic control.

**4.1 Dietary Assessment.** Three-day food records are routinely collected as part of patient care for individuals with PKU and MCAD, and patients’ families have previously been instructed on keeping accurate diet records as part of their care. The controls recruited from Kindering and the expanded controls were provided with printed instructions on how to maintain an accurate diet record. The participants were asked to complete the three-day diet record within one week of obtaining the fecal sample. We analyzed the diet records for total energy, macronutrients, including grams per day and percent of total calories of carbohydrate, protein and fat, PHE intake, and dietary fiber using Nutrition Data System for Research (NDSR, version 2012). To acknowledge the age range of our study group and that there are differences in total energy based on age and weight we further calculated energy per kilogram of body weight for each study participants to compare the intake of the two groups. The prebiotic content (e.g., oligosaccharides) content of the diet was hand calculated based on breast milk and formula intake and published composition of each.

**4.2 Fecal Specimen Collection and Analysis.** Fresh fecal samples were collected directly into RNAlater to preserve the bacterial DNA as described previously (43). DNA was extracted from stool stored in RNAlater at -80°C (44). The 16S rRNA gene was amplified and sequenced using 454 pyrosequencing primers 27f and 519 reverse (40, 45-50) for amplicon pyrosequencing (bTEFAP) (46) at Research and Testing (Shallowater, TX) utilizing Roche 454 FLX titanium instruments and reagents and following manufacturer’s guidelines.

Sequences were converted to standard FASTA format. Briefly, sequences were removed if they were <300 bp, had homopolymers >8 bp, with no exact match to the forward primer or a barcode, and
did not align to the appropriate 16S rRNA gene region. In the remaining pool, sequences were trimmed from the 3’ end until they had an average quality score of 35 using a moving window of 50 bp. Sequences were aligned and identified in MOTHUR (v.1.28.0) to the Silva 16S rRNA gene reference alignment (www.arb-silva.de) (51-53). Potentially chimeric sequences were removed using ChimeraSlayer (54). The pre.cluster option in MOTHUR was used to minimize the effect of pyrosequencing errors in overestimating microbial diversity (55). Sequences were grouped into operational taxonomic units (OTU) using the mean Neighbor-Joining algorithm. OTU were defined by clustering at 3% divergence (97% similarity) and used for diversity estimates. The Bayesian algorithm classification was used for phylogenetic classification of each sequence with a minimum bootstrap value of 60% (49). Bacterial taxa that were less than 0.036% of the total sequences in a sample were removed. The number of sequences in each genera was converted to arcsin relative percent for multivariate analysis.

5. Statistical Analysis

Aims 1 and 2. To compare the gut microbial profile of infants and toddlers with PKU receiving the majority of their nutrition from metabolic formula to sex- and age-matched individuals with MCAD (controls), who receive conventional dietary treatment. **Rationale:** The composition of the GMC will differ between individuals with PKU compared to those receiving conventional diets.

**Statistical approach:** First, we defined a summary composite measure of microbial species using NMS. Next, we applied a mixed model, including additional terms to adjust for demographic confounders. Finally, to test for significance among specific sets of microbial species, a follow-up multi-response permutation procedure (MRPP) test was performed on the sub-communities most highly correlated with the dominant few NMS axes.

We compared the gut microbial parameters and the diets of PKU patients as compared to controls. The statistical model used to evaluate the GMC may be written in the following form:

\[ Y_j = \alpha + \beta_1 W_j + \beta_2 X_j + E_j, \quad j=1,\ldots,n \]
where \( Y_j \) is GMC composition measured using pyrotag sequencing or dietary parameters, \( W_j \) is PKU patients and controls, \( X \) is a vector of relevant covariants (such as participants’ sex, long-term antibiotic use, etc) and \( E_j \) is the Gaussian error term. The null hypothesis \( H_0: \beta_1=0 \) is used to test whether the mean of each microbial parameter or the dietary constituent differs between the 2 study populations.

**Aim 3.** To quantify and compare the prebiotic content of the diets of infants and toddlers with PKU to the diets of controls via detailed diet analysis. **Rationale:** The prebiotic content (e.g., dietary fiber, resistant starch, and oligosaccharides) will differ between the diets of PKU patients as compared to those receiving conventional diets.

**Statistical approach:** The null hypothesis \( H_0: \beta_1=0 \) is used to test whether the mean of each microbial parameter or the dietary constituent differs between the 2 study populations.

The categorical demographic information that was obtained from the questionnaire was consolidated and compared with Fisher’s exact test (two tailed) using GraphPad, an on-line statistical calculator (http://graphpad.com/quickcalcs/contingency1). The numerical data (age, weight, height) collected from the questionnaire as well as the diet analysis results were compared by t-tests calculated by Microsoft Excel for Mac 2011. Microsoft Excel for Mac 2011 was also used to calculate the reported data averages and standard deviations.

**Results**

**Subject recruitment:**

At the time of the initial IRB submission for this study there were 16 individuals with PKU who were younger than 3 years and were followed monthly at the Cristine M. Trahms Program for Phenylketonuria at the University of Washington. Once the IRB approval was obtained, the eligible PKU population had decreased from 16 individuals to 9 eligible subjects. Based on historic averages, we anticipated an additional 5 individuals per year to be identified with PKU through the Washington State Newborn Screening program; however, during the study recruitment
efforts from September 2013 until December 2013 there were not any additional individuals diagnosed with PKU through the newborn screening program. Six individuals with PKU participated in this study, which represented 66.7 percent of eligible individuals with PKU.

Control recruitment:

MCAD deficiency controls:

At the time of the initial IRB submission, the UW Program followed 12 individuals with MCAD deficiency, however there were only 6 eligible study participants at the time of active subject recruitment. Based on historic averages, we anticipated an additional 5 individuals to be diagnosed with MCAD deficiency annually; however, during the study recruitment efforts from September 2013 until December 2013, there were not any additional individuals diagnosed with MCAD deficiency through the newborn screening program. We were able to recruit 2 individuals with MCAD deficiency, which represented 33.3 percent of eligible individuals with MCAD deficiency.

Kindering Controls:

Two individuals from Kindering also participated as controls. There was very poor subject recruitment from the published study announcement in the monthly Kindering newsletters; only one control subject was obtained though the announcement. Three class outreach sessions were held. The first class had 2 subjects enroll out of a potential 4 subjects, the second class outreach had zero interest out of 3 possible subjects, and the final class outreach had 2 subjects enroll out of 6 potential subjects. Overall 4 out of a potential 13 individuals agreed to participate and were consented from the Kindering class outreach sessions, however only 2 participants completed the study activities.

Expanded Controls:

Due to low interest from the initially selected control groups, on January 8, 2014 an IRB modification was approved to expand the recruitment population for the control subjects by sending study announcements via e-mail to departmental list serves and placing flyers in community centers. There was an excellent response to the modified recruitment strategies and by the end of February 2014 the additional controls had completed the study activities.
Demographics:

There were no statistically significant differences between the age, gender or anthropometric characteristics of the participants with PKU compared to controls as summarized in Table 1. Average age (in months) of participants with PKU was 18.1 ± 10.3 and average age for controls was 21.1 ± 9.5. The questionnaire showed no statistically significant differences in general diet characteristics between the two participant groups (Table 2). The majority of participants were provided with some breast milk; 100% of the participants with PKU had received breast milk and 92.3% of the controls had received breast milk (Table 2). There were no differences between the group for age of weaning or prebiotic and/or probiotic supplementation use (Table 2).

The questionnaire that was administered to identify other variables between participants with PKU and controls that may impact the GMC showed that there were no statistically significant differences between the two groups. The maternal characteristics (Table 3) and the birth conditions (Table 4) of the two participant groups showed no statistically significant differences. In addition, antibiotic use and general characteristics of the two groups show no statistically significant differences (Table 5).

PHE concentrations:

The chart review of individuals with PKU showed that all patients were in good metabolic control; with average PHE concentrations over the past 12 months (or lifetime for individuals with PKU that were less than 1 year of age) were within normal treatment range (Table 6).

Diet Analysis:

The dietary assessment of the participants with PKU compared to the controls, as summarized in Table 7, revealed no statistical difference between total energy intake (kcal/d), total carbohydrate intake (g/d) or fat intake (g/d). As expected there was a statistically significant difference in the protein (p=0.02) and phenylalanine (p<0.001) intake between the two groups. There was also a significant difference (p<0.001) in the dietary fiber intake (g/d) in the participants with PKU (3.66 ± 3.59) compared to controls (9.2 ± 5.7). The oligosaccharide intake was estimated, however there was an inadequate
number of participants receiving breast milk or standard infant formula at the time of the study to determine whether any significant differences between the two groups existed. At the time of the study only one participant with PKU was taking breast milk, which provided an estimated 3.9 g/d of oligosaccharides, and one participant with PKU was taking standard infant formula, which provided an estimated 1.9 g/d of oligosaccharides, compared to the control group where 3 participants were taking breast milk, which provided an estimated 9.5 ± 7 g/d of oligosaccharides and 3 participants were being provided with standard infant formula which provided an estimated 2.2 ± 1.4 g/d of oligosaccharides.

**Gut Microbial Community:**

Using 454 pyrosequencing of the V1-V3 region, a total of $5.1 \times 10^5$ raw sequences were processed using QC standards. The resulting pool of 420700 sequences, which averaged 4382 ± 3351 sequences per study participant, was analyzed. The sequences were, on average, 365 bp long. The trimmed sequences represented a total of X bacterial genera and, on average, 724 ± 287 OTUs generated. Bacteria were distributed across 5 phyla: Firmicutes (57%), Bacteroidetes (37%), Proteobacteria (2.3%), Verrucomicrobia (0.4%), and Actinobacteria (2.7%). There was no significant difference in the distribution of bacterial phyla between control and PKU subjects (Figure 1 and 2). Figures 3a and 3b show the relative abundance of bacterial genera by sex in subjects with and without PKU. There was no significant difference in the Shannon diversity index, which is a measure of the evenness and richness of the GMC within a person, between non-PKU (Shannon diversity= 5.4 +/- 1.1) and PKU (Shannon diversity=4.8 +/-1.2; Tukey’s, p=0.16) subjects (Figure 4). The percent similarity of the GMC in the study participants do not cluster by PKU (Figure 5a), however the is an observable cluster by age (Figure 5b).

**Discussion**

As with any pilot study our statistical power was limited, however the information from this pilot study generated important preliminary data that can be used to support a future grant submission for a larger study.
This pilot study demonstrated that families of patients with PKU are highly motivated to participate in research protocols. Recruitment yield from the 9 eligible patients with PKU followed at the University of Washington PKU clinic was 66.7%. On the other hand, recruitment of control patients resulted in a significantly lower subject yield. Alternative recruitment methods of controls may be considered in the future study efforts to improve yields. Since in-person presentations and direct contacts achieved best subject recruitment yields, in-person study presentations to various community new parent groups may be a more effective strategy for future recruitment of controls.

Our group of participants with PKU was comparable to our control group as evaluated by the study questionnaire. There were significant dietary differences between the two groups, specifically, there were significant differences in protein, PHE and fiber between individuals with PKU compared to controls, however none of the intakes of the two groups were outside expected intake for age. The oligosaccharide intake of participants with PKU appeared to be lower than that of controls, however our limited number of data points for comparison failed to support any conclusive results. Further diet analyses and examination of oligosaccharide intake in individuals with PKU is warranted.

Several factors may explain why we were unable to detect any differences in the GMC of participants with PKU compared to controls. First, our sample size was limited. Previous studies of GMC in healthy adults have revealed gut microbial communities that were highly variable both within and between people (56). Our study also revealed that there is high variability of the GMC in both the infants and toddlers with PKU and the controls, consistent with other studies that show interpersonal variation in gut microbial diversity is greater between infants than between adults (57). It is likely because of this variability that we didn’t see any differences between the two study groups at the genera level. Knowing that the GMC is highly variable, more study participants would likely be required to see any significant collective differences between the individuals with PKU compared to controls.

Second, there are many factors that impact the GMC, including introduction of solid foods (58). Our study sample included both individuals that were on formula or breast milk only and those who had been weaned and were receiving a conventional toddler diet. As infants are weaned, pancreatic
function, small intestinal absorption and colonic fermentation capacity mature, which changes the characteristics of colonic material and the microbiota composition (59). A two and a half year case study of the assembly of the human infant gut microbiome showed that ingestion of table foods resulted in a sustained increased in the abundance of Bacteroidetes, as well as increased fecal short chain fatty acid levels, enrichment of genes associated with carbohydrate metabolism, vitamin biosynthesis and a more stable community composition, which is a shift towards the characteristics of the adult microbiome (58).

The fact that we were able to see differences in clustering by age may have been a reflection of the greater diet variety in the older children and resulting GMC changes seen with weaning. In the future, comparisons of GMC in participants with PKU and controls prior to introducing solid foods in the diet may provide more specific insight into the nutritional differences of infant metabolic formulas without oligosaccharides compared to controls that receive a conventional diet. Separate comparisons of older children with PKU compared to controls would still be of interest, and a study design with more stringent parameters around participants’ age and other variables may provide more insight into the dietary differences that impact GMC.

This study showed that the gut bacteria were distributed predominantly as Firmicutes (57%) and Bacteroidetes (37%), which is a similar finding to study of healthy European children aged 1-6 years which showed a gut bacterial distribution of Fimicutes (64%) and Bacteriodetes (22%) (60). Our findings are somewhat different from previous published data of healthy Canadian infants that showed a predominance of Firmicutes (44%) and Actinobacteria (36%), as opposed to Bacteriodetes, however the Canadian study examined 4 month old infants only and did not include the expanded age range of our population (61).

In order to achieve an adequate number of participants for a more appropriately powered study a multi-center approach would likely be required. Alternatively, designing a study over an extended time period would allow for increased enrollment and may also provide some opportunities for multiple fecal samples to be obtained and provide greater insight into the GMC of these individuals over time.
Conclusion

This pilot study provides important groundwork for future investigation of the GMC in individuals with PKU. This study demonstrated that there are significant dietary differences in protein, phenylalanine and fiber between individuals with PKU compared to controls. Further studies to quantify the oligosaccharide intakes of individuals with PKU are warranted. There are no statistically significant differences in the GMC of individuals with PKU compared to controls, although total bacterial diversity tended to be less in individuals with PKU. Nutrition therapy is the cornerstone for the medical management of PKU and clinicians must continue to be vigilant to address all factors that may affect treatment outcomes, including the development of an optimal GMC (31). Understanding the GMC of individuals with PKU may help guide the development of nutritional products that may support health-promoting microbiota, which could contribute to overall wellness of this patient population. This exploratory study indicates that further investigation is warranted in trials with larger numbers to fully evaluate the GMC in individuals with PKU compared to controls.
Acknowledgements

This project would not have been possible without the generous funding provided by Nutricia North America, the Pediatric Nutrition Practice Group (PNPG) of the Academy of Nutrition and Dietetics (AND) and the University of Washington Nutritional Sciences Program. This project also was supported by Alanna Boynton, MS, RD at the Nutrition Shared Resource at the Fred Hutchinson Cancer Research Center and by Meredithullar, PhD, Lisa Levy, MS, RD and Lauren Hunter at the Fred Hutchinson Cancer Research Center. I would also like to thank my committee chair, Johanna Lampe, PhD, RD who was an incredible support and mentor; and my committee member, Beth Ogata, MS, RD who provided much motivation and encouragement that kept me on track. Many thanks also to the staff at the Cristine M. Trahms Program for Phenylketonuria at the University of Washington, including Dr. C. Ron Scott, MD, Janie Heffernan, MS, RD, Mari Obara, MS, RD and Vicki Frasher for their support of this project and their dedication to individuals with PKU and other inborn errors of metabolism. Lastly, I would like to thank my husband, Brian Gunnarson, for being my kite string and keeping me grounded on this extended educational adventure.
Table 1 – Demographics of Infants and Toddlers with and without PKU

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>Subjects with PKU (n=6)</th>
<th>Controls (n=13)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (in months)</strong></td>
<td></td>
<td>18.1 ± 10.3</td>
<td>21.1 ± 9.5</td>
<td>0.52</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td>3 (50%)</td>
<td>6 (46.2%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>3 (50%)</td>
<td>7 (53.9%)</td>
<td></td>
</tr>
<tr>
<td><strong>Birth weight (in grams)</strong></td>
<td></td>
<td>3404 ± 566</td>
<td>3270 ± 464</td>
<td>0.59</td>
</tr>
<tr>
<td>Weight for age %ile</td>
<td></td>
<td>56 ± 34</td>
<td>47 ± 28</td>
<td>0.49</td>
</tr>
<tr>
<td><strong>Birth Length (in cm)</strong></td>
<td></td>
<td>50.5 ± 2.3</td>
<td>50.3 ± 2.4</td>
<td>0.88</td>
</tr>
<tr>
<td>Length for age %ile</td>
<td></td>
<td>62 ± 29</td>
<td>61 ± 34</td>
<td>0.94</td>
</tr>
<tr>
<td>**Birth weight for length %ile</td>
<td></td>
<td>39 ± 43</td>
<td>28 ± 33</td>
<td>0.59</td>
</tr>
<tr>
<td><strong>Current weight (in kg)</strong></td>
<td></td>
<td>11.5 ± 3.1</td>
<td>10.7 ± 2.8</td>
<td>0.59</td>
</tr>
<tr>
<td>Weight for age %ile</td>
<td></td>
<td>69 ± 28</td>
<td>45 ± 32</td>
<td>0.14</td>
</tr>
<tr>
<td>**Current length/ height (in cm)</td>
<td>84.2</td>
<td></td>
<td>80.7</td>
<td>0.62</td>
</tr>
<tr>
<td>Length/ height for age %ile</td>
<td></td>
<td>59 ± 34</td>
<td>68 ± 34</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=3)</td>
<td>(n=9)</td>
<td></td>
</tr>
<tr>
<td><strong>Current weight for length/ height</strong></td>
<td>68 ± 20</td>
<td></td>
<td>46 ± 40</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=3)</td>
<td>(n=9)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 – Diet Characteristics of Infants and Toddlers with and without PKU

<table>
<thead>
<tr>
<th></th>
<th>Number of responses</th>
<th>Subjects with PKU (n=6)</th>
<th>Controls (n=13)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breast fed</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0</td>
<td>1 (7.7%)</td>
<td>12 (92.3%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Yes</td>
<td>6 (100%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age of weaning if breast fed</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than 9 months</td>
<td>2 (33.3%)</td>
<td>4 (30.8%)</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Greater than 9 months</td>
<td>3 (50%)</td>
<td>5 (38.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Does not apply, current breast milk</td>
<td>1 (16.7%)</td>
<td>3 (23.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Formula fed</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0</td>
<td>5 (38.5%)</td>
<td></td>
<td>0.13</td>
</tr>
<tr>
<td>Yes</td>
<td>6 (100%)</td>
<td>8 (61.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Child probiotic use</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never/ sporadic</td>
<td>6 (100%)</td>
<td>10 (76.9%)</td>
<td></td>
<td>0.52</td>
</tr>
<tr>
<td>Used</td>
<td>0</td>
<td>3 (23.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Child prebiotic use</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never/ sporadic</td>
<td>6 (100%)</td>
<td>12 (92.3%)</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Used</td>
<td>0</td>
<td>1 (7.7%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 3 – Maternal Characteristics of Infants and Toddlers with and without PKU

<table>
<thead>
<tr>
<th>Maternal characteristic</th>
<th>Number of responses</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subjects with PKU (n=x)</td>
<td>Controls (n=13)</td>
<td>P value</td>
<td></td>
</tr>
<tr>
<td>Maternal probiotic use</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never/sporadic Use</td>
<td>4 (66.7%)</td>
<td>11 (84.6%)</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>Use</td>
<td>2 (33.3%)</td>
<td>2 (15.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal prebiotic use</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never/sporadic Use</td>
<td>5 (83.3%)</td>
<td>12 (92.3%)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Use</td>
<td>1 (16.7%)</td>
<td>1 (7.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regular</td>
<td>5 (83.3%)</td>
<td>8 (61.5%)</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>1 (16.7%)</td>
<td>5 (38.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal Education</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than college graduate</td>
<td>1 (16.7%)</td>
<td>2 (15.4%)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>College graduate or higher</td>
<td>5 (83.3%)</td>
<td>11 (84.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal antibiotic use during this pregnancy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>6 (100%)</td>
<td>9 (69.2%)</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0</td>
<td>4 (30.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal vaccination status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All vaccines</td>
<td>6 (100%)</td>
<td>13 (100%)</td>
<td>n/a</td>
<td></td>
</tr>
</tbody>
</table>

### Table 4 – Birth Conditions of Infants and Toddlers with and without PKU

<table>
<thead>
<tr>
<th>Birth condition</th>
<th>Number of responses</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subjects with PKU (n=x)</td>
<td>Controls (n=x)</td>
<td>P value</td>
<td></td>
</tr>
<tr>
<td>Maternal rupture of membranes</td>
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<td></td>
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</tr>
<tr>
<td>Less than 24 hours</td>
<td>6 (100%)</td>
<td>13 (100%)</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>Place and mode of delivery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal births</td>
<td>3 (50%)</td>
<td>8 (61.5%)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Cesarean section</td>
<td>3 (50%)</td>
<td>5 (38.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hospitalization after birth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than 2 days</td>
<td>4 (66.7%)</td>
<td>10 (76.9%)</td>
<td>1.00</td>
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</tr>
<tr>
<td>2 days or more</td>
<td>2 (33.3%)</td>
<td>3 (23.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant’s Gestational age at birth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than 37 weeks (preterm)</td>
<td>1 (16.7%)</td>
<td>2 (15.4%)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>37 or more</td>
<td>5 (83.3%)</td>
<td>11 (84.6%)</td>
<td></td>
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</table>
Table 5 – Antibiotic use and General Characteristics of Infants and Toddlers with and without PKU

<table>
<thead>
<tr>
<th></th>
<th>Number of responses</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subjects with PKU (n=6)</td>
<td>Controls (n=13)</td>
<td>P value</td>
<td></td>
</tr>
<tr>
<td>Fever in first month of life</td>
<td>6 (100%)</td>
<td>13 (100%)</td>
<td>n/a</td>
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<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibiotic Use</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3 (50%)</td>
<td>7 (53.9%)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3 (50%)</td>
<td>6 (46.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibiotic use more than once</td>
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<td></td>
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<tr>
<td>No</td>
<td>4 (66.7%)</td>
<td>10 (76.9%)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2 (33.3%)</td>
<td>3 (23.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibiotic use in first month</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>of life</td>
<td>6 (100%)</td>
<td>13 (100%)</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaccinated per pediatrician</td>
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<td></td>
<td></td>
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<tr>
<td>recommendations</td>
<td>0</td>
<td>3 (23.1%)</td>
<td>0.52</td>
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</tr>
<tr>
<td>No</td>
<td>6 (100%)</td>
<td>10 (76.9%)</td>
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<tr>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Siblings</td>
<td></td>
<td></td>
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<tr>
<td>None</td>
<td>5 (83.3%)</td>
<td>5 (38.5%)</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1 (16.7%)</td>
<td>8 (61.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Live on farm or have livestock</td>
<td>6 (100%)</td>
<td>13 (100%)</td>
<td>n/a</td>
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<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Furry pets in home</td>
<td></td>
<td></td>
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<tr>
<td>None</td>
<td>2 (33.3%)</td>
<td>7 (53.9%)</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4 (67.7%)</td>
<td>6 (46.2%)</td>
<td></td>
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</tbody>
</table>

Table 6 – Summary of Phenylalanine levels (mg/dL) in Participants with PKU (past 12 months)

<table>
<thead>
<tr>
<th>Subject Number</th>
<th>Mean ± SD</th>
<th>Minimum</th>
<th>Maximum</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>2.6 ± 1.0</td>
<td>1.1</td>
<td>4.9</td>
<td>13</td>
</tr>
<tr>
<td>102</td>
<td>2.8 ± 2.3</td>
<td>0.1</td>
<td>8.7</td>
<td>26</td>
</tr>
<tr>
<td>103</td>
<td>3.8 ± 3.4</td>
<td>0.2</td>
<td>16.6</td>
<td>35</td>
</tr>
<tr>
<td>104</td>
<td>4.5 ± 4.4</td>
<td>0.2</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>112</td>
<td>3.4 ± 3.4</td>
<td>0.4</td>
<td>11.6</td>
<td>15</td>
</tr>
<tr>
<td>115</td>
<td>2.5 ± 1.0</td>
<td>1.2</td>
<td>4.5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Subjects with PKU (n=6)</td>
<td>Controls (n=13)</td>
<td>P value</td>
</tr>
<tr>
<td>------------------------------</td>
<td>----------------------------</td>
<td>-------------------------</td>
<td>-----------------</td>
<td>---------</td>
</tr>
<tr>
<td>Total energy (kcal/d)</td>
<td></td>
<td>991 ± 223</td>
<td>956 ± 295</td>
<td>0.65</td>
</tr>
<tr>
<td>kcal/ kg body weight</td>
<td></td>
<td>89 ± 19</td>
<td>83 ± 20</td>
<td>n/a</td>
</tr>
<tr>
<td>Carbohydrate (g/d)</td>
<td></td>
<td>127 ± 48</td>
<td>123 ± 45</td>
<td>0.79</td>
</tr>
<tr>
<td>% of energy</td>
<td></td>
<td>50 ± 8</td>
<td>51 ± 9</td>
<td>0.63</td>
</tr>
<tr>
<td>Dietary fiber (g/d)</td>
<td></td>
<td>3.7 ± 3.6</td>
<td>9.2 ± 5.7</td>
<td>0.0004</td>
</tr>
<tr>
<td>Oligosaccharides (g/d)</td>
<td></td>
<td>3.9 (n=1)</td>
<td>9.5 ± 7 (n=3)</td>
<td>n/a</td>
</tr>
<tr>
<td>From human milk</td>
<td>1.9 (n=1)</td>
<td>2.2 ± 1.4 (n=3)</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>From standard formula</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (g/day)</td>
<td></td>
<td>26 ± 4</td>
<td>35 ± 17</td>
<td>0.02</td>
</tr>
<tr>
<td>% of energy</td>
<td></td>
<td>11 ± 2</td>
<td>12 ± 5</td>
<td>0.005</td>
</tr>
<tr>
<td>Phenylalanine (mg/d)</td>
<td></td>
<td>279 ± 101</td>
<td>1634 ± 810</td>
<td>3.09x10^{-9}</td>
</tr>
<tr>
<td>Fat (g/d)</td>
<td></td>
<td>44 ± 6</td>
<td>38 ± 15</td>
<td>0.081</td>
</tr>
<tr>
<td>% of energy</td>
<td></td>
<td>41 ± 6</td>
<td>36 ± 11</td>
<td>0.11</td>
</tr>
</tbody>
</table>
Figure 1. Distribution of bacteria phyla between control and PKU subjects.

Figure 2. Distribution of bacterial phyla in study subjects
Figure 3. The distribution of bacteria genera. Genera that are in bold are the most dominant or of particular interest.
Figure 4. Shannon diversity estimates between control and PKU subjects.
Figure 5a. Subjects do not cluster by PKU (subjects with PKU in grey, controls in black)

Figure 5b. Subjects cluster by age (subjects > 1 year in grey, subjects <1 year in black)
REFERENCES


**Gut Microbial Community in Infants and Toddlers with and without Phenylketonuria (PKU) Study Questionnaire**

1. I am the child’s ____________ who is answering the questionnaire.
   a. Mother
   b. Father
   c. Other  Please indicate: ________________________

2. Your child’s birthday _____ year ______month _______day

3. Gender of your child
   a. Male
   b. Female

4. What kind of diet does the mother of your child eat?
   a. Regular
   b. Organic
   c. Vegetarian
   d. Other _______________

5. Does the mother of your child use probiotic supplements (bacterial supplements or foods with live active cultures such as Lactobacillus or Bifidobacterium)?
   a. Never/ sporadic
   b. Several times per month
   c. Several times per week
   d. Daily

6. Does the mother of your child use prebiotic supplements (non digestible food supplements such as fiber supplements, fructooligosaccharides (FOS), or inulin)?
   a. Never/ sporadic
   b. Several times per month
   c. Several times per week
   d. Daily

7. What is the highest level of education of the mother of your child?
   a. Did not graduate high school
   b. High school graduate
   c. Vocational school graduate (associate degree)
   d. College graduate (bachelor degree)
   e. Graduate degree or higher

8. Did the mother use antibiotics during pregnancy with this child?
   a. No
   b. Yes  If yes, were antibiotics use during the last month of pregnancy with this child?
      i. No
      ii. Yes

9. Is the mother of the child vaccinated against common childhood illnesses?
   a. Mother had all the vaccinations that were common for her age
   b. Mother only had DTP (Diphtheria, pertussis, tetanus, polio)
   c. Mother was not vaccinated as a child but has had catch-up vaccinations
   d. Unknown
   e. Mother had no vaccinations  if so what was the reason for this?
      i. Belief
      ii. Concerns for other health risks
      iii. Other, namely ______________
10. How long was the time between mother’s rupture of membranes and delivery of your child?
   a. Less than 24 hours
   b. Greater than 24 hours

11. Place and mode of your child’s delivery
   a. Natural delivery at home
   b. Natural delivery in hospital
   c. Natural birth with pain management (spinal, epidural in the hospital)
   d. Assisted delivery (vacuum extraction, forceps delivery, etc)
   e. Cesarean section in hospital
   f. Other, namely ____________________________

12. How many days was your baby hospitalized after birth?
   a. None (home birth)
   b. Less than 24 hours
   c. 1-2 days
   d. 2-3 days
   e. 4-6 days
   f. More than 7 days

13. What was the gestational age at birth of your child?
   a. Less than 37 weeks (premature)
   b. 37-41 weeks (term)
   c. Greater than 41 weeks (postmature)

14. What was your child’s BIRTH weight?
   a. __________ pounds __________ ounces

15. What was your child’s BIRTH length?
   a. __________ inches

16. What is your child’s CURRENT weight?
   a. __________ pounds

17. What is your child’s CURRENT length/ height?
   a. __________ inches

18. Did your child experience fever in the first month of life (not including fever from vaccinations)?
   a. No
   b. Yes; If yes, what was the highest temperature?
      i. Less than 38 degrees
      ii. 38 to 38.9 degrees
      iii. 39 degrees or higher
      iv. Do no know/ not measured

19. Has your baby ever had to take any of the following antibiotic medicines? If so, for how long?

<table>
<thead>
<tr>
<th>Medicine</th>
<th>Never taken</th>
<th>Taken 1-3 days</th>
<th>Taken 4-10 days</th>
<th>Over 10 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin (syrup/ liquid)</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Augmentin</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Other; namely</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
</tr>
</tbody>
</table>
20. Has your child been prescribed antibiotics more than once?
   a. No
   b. Yes → If yes, how many times?
      i. Twice
      ii. Three times
      iii. More than three times

21. Did your child require antibiotics during first month of life?
   a. No
   b. Yes

22. Is your child vaccinated according to their pediatrician’s recommendations?
   a. No
   b. Yes

23. Is (or was) your child breast-fed/ or provided with breast milk?
   a. No → If no, skip to question 25
   b. Yes

24. If yes to above, at what age was your child weaned from breast milk?
   a. Less than 4 months
   b. 4-6 months
   c. 6-9 months
   d. 9-12 months
   e. 12-18 months
   f. over 18 months
   g. Does not apply; my child is still breast feeding

25. Is (or was) your child formula fed?
   a. No
   b. Yes; If yes, what is (or was) your child’s MAIN formula?
      _________________________________

26. Does your child use probiotic supplements (bacterial supplements or foods with live active cultures such as Lactobacillus or Bifidobacterium)?
   a. Never/ sporadic
   b. Several times per month
   c. Several times per week
   d. Daily (almost daily)

27. Does your child use prebiotic supplements (non digestible food supplements such as fiber supplements, fructooligosaccharides (FOS), or inulin)?
   a. Never/ sporadic
   b. Several times per month
   c. Several times per week
   d. Daily (almost daily)

28. Does your child have siblings?
   a. None
   b. 1
   c. More than 2 siblings

29. Do you and your child live on a farm or have livestock?
   a. No
   b. Yes

30. Are there furry pets in your child’s home?
   a. None
   b. Dog
   c. Cat
   d. Other ____________________