Effects of dietary protein intake on fecal and milk microbiota

by

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ABSTRACT

Microorganisms in milk have traditionally been assumed to be contaminants, but recent data suggest that these microbial communities naturally reside in milk and may contribute to vital maternal effects. Investigators have speculated that microorganisms are derived, at least in part, from populations of microorganisms found in a mother's gut. Milk microorganisms are ingested by offspring gut and contribute to the microbial populations that colonize the offspring gut. Thus, factors that impact the population of microorganisms in milk have implications for advancing knowledge of how mothers influence offspring development. In this case, females are likely preparing offspring for a similar dietary environment at independence. To characterize the relationship between milk microbial diversity and maternal protein intake, a pilot study was conducted to characterize impact of maternal protein intake on gut microbial diversity in rats. Based on this preliminary analysis, diets with 10% and 20% protein were selected to determine impact of protein intake on milk microbial diversity.

Milk was collected from Sprague-Dawley rat dams at 14 days post-partum. No differences were observed for dry matter or crude protein content of milk between treatment groups. Alpha diversity of milk microbiota from high protein fed rats was greater than low protein fed rats. *Staphylococcus* spp. taxa relative abundance was higher in LP milk samples compared to HP samples and *Lactobacillus* spp. taxa had higher relative abundance in HP milk samples compared to LP samples. The results of this investigation indicates that dietary protein intake affects gastrointestinal microbial diversity and suggests that protein content of a mother's diet impacts relative abundance of milk microorganisms. This study highlights the importance of

dietary protein intake on composition of rat fecal and milk microbiota, with higher protein intake favoring potentially beneficial *Lactobacillus* populations in milk and reducing the relative abundance of potential pathogens like *Staphylococcus* taxa. *Proteus mirabilis* and *Enterococcus faecalis* were cultured from rat milk and are likely natural occurring bacteria found in rat milk. Future work with milk microorganisms involves examining possible interactions between beneficial bacteria like *Lactobacillus* and potential pathogenic bacteria like *Staphylococcus* to examine how maternal diet can affect offspring development.

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List of Abbreviations

ANOSIM Analysis of Similarities

CFU   Colony forming unit

gDNA   Genomic DNA

HP   High-protein diet

LP   Low-protein diet

MP   Moderate-protein diet

MSA   Mannitol salt agar

OTU   Operational Taxonomic Unit

PCoA   Principal Coordinate Analysis

QIIME   Quantitative Insights Into Microbial Ecology

SM   Skim milk agar

SPF   Specific-pathogen free

TSA   Tryptic soy agar

INTRODUCTION

Milk is a product of mammalian evolution that provides offspring with lipids and proteins essential for growth, antimicrobial compounds that inhibits pathogens, maternal antibodies that confer passive immunity, and a complex community of microbiota (1-4). Microorganisms in milk were originally assumed to be contaminants, but recent data suggest a microbial community naturally resides in a mother's milk (5-7). These microorganisms are derived from populations residing on a mother's breast or teat skin, offspring mouths, and environment (8-11). Functional significance of milk microbial communities is an active area of research. As microorganisms ingested via milk contribute to incipient microbial populations that populate the offspring gut, factors impacting this community may have important implications for advancing knowledge of maternal effects on offspring development.

Food that an animal consumes can have significant impacts on microorganisms taking residence in an individual's digestive tract (12). Work by Perez *et al.* (13) has shown that microorganisms in milk may be derived, in part, from microorganisms that are transported by mucosal dendritic cells of the mother's immune system from the mother's gastrointestinal tract (14). However, more studies are needed to confirm this finding and to understand the mechanisms of bacterial translocation from gut to mammary gland (15). Regardless of whether or not gut microorganisms are directly transported to the mammary gland, mother's diet does appear to influence both the community of microorganisms present in a mother's gut and her milk (16-18). Goldsmith *et al.* (19) reviewed how microorganisms in a mother's milk that contribute to offspring development. Transferred microorganisms colonize an offspring's gut and

those microorganisms are necessary for breaking down certain food components (20). Gut microorganisms influence what kinds of food offspring may efficiently consume when they are weaned is well documented with ruminant animals (21, 22). Microorganisms transferred from mother to offspring through milk appear to serve an important maternal effect, contributing to offspring phenotype, performance, and survival.

The goal of this investigation is to evaluate impacts of dietary protein intake on gut microbial diversity and in turn, the microbial community occurring in a mother's milk. Dietary protein intake impacts milk composition and offspring development because higher protein intake has been shown to increase protein content of a mother's milk (4, 23, 24). Offspring require protein for growth and immune functions (25-29). Immune development is impacted by offspring's protein intake from milk (30, 31). Not meeting protein requirements can lead to an underdeveloped immune system, impairing gut microbiota colonization and reduce recognition of beneficial microorganisms by gut immune cells, and further complications with growth that can occur later in life due to being immunocompromised (13, 30, 32). It is probable that maternal protein intake will also impact the community of microorganisms found in a mother's milk and ultimately, colonize the gut of her young. High-protein diets shift microbial community in the consumer's intestine and affect milk composition and production (33). It is also possible that gut microorganisms in offspring are adapted to a relative protein intake similar to a mother's relative intake which can be necessary for offspring development (21). However, there is limited research done on how dietary protein intake can affect milk microorganisms (33, 34). There may be a biological significance on how dietary protein intake affects milk composition and milk microbiota. Studying dietary protein intake effects on milk microbiota in rats may provide

insights into better understanding of proteins' role on growth and development through host-microbe interactions.

First, I conducted a pilot study with female rats to determine if relative content of a rat's diet affected gut microbiota. Preliminary results confirmed that dietary protein intake affects fecal microbial diversity with rats consuming 10% or 15% protein diets, displaying similar fecal microbial diversity, but rats consuming the 10% protein diet showed a greater difference in fecal microbial diversity when compared to rats consuming the 20% protein diet. A second study was designed with using diets with 10% and 20% protein to determine impact of protein intake on milk microbial diversity. This study addresses two questions: 1. Does dietary protein intake affect gut microbial diversity? 2. Does dietary protein affect milk microbial diversity? Rats consuming low-protein (LP) diet were predicted to have significantly less gut diversity of microorganisms associated with reduced protein availability and shift of gut microbial populations to microorganisms that utilize carbohydrates as a primary nutrient source. Dietary protein intake is also predicted to affect what bacterial species are found in feces and milk.

MATERIALS AND METHODS

Auburn University's Institutional Animal Care and Use Committee, protocol number 2014-2544, approved all methods for this study.

Common Animal and Housing Conditions

Sprague-Dawley rats (*Rattus norvegicus*) were selected for this study because of their docility, large litters, and strong maternal instinct (35-37). Their small size makes them easy to house in large numbers for stronger statistical power and they are relatively inexpensive to

maintain (36). Rat dams also produce copious amount of milk relative to smaller rodents, allowing investigators to measure multiple variables from a single sample. Sprague-Dawley rats obtained from Harlan/Envigo Laboratories (Indianapolis, IN) were used for all three experiments. Rats were housed at Auburn University's Biological Research Facility under specific pathogen-free and standard housing conditions (14:10 light cycle, 21 °C). All females were housed in pairs and males were housed individually except when females were moved into their cages for mating. Data collection was limited to females in all experiments.

Three different custom rodent diets that varied in relative protein content, low-protein (LP = 10%), moderate-protein (MP = 15%), and high-protein (HP = 20%), were obtained from TestDiet (Purina Animal Nutrition, LLC., St. Louis, MO). The amount of cornstarch in each diet was adjusted to ensure each diet was isocaloric, but diets were otherwise similar (Table 1). All three diets were plant-based in effort to feed the rats a diet that had greater similarity to their natural diet than the milk protein rich diets commonly fed to laboratory rodents. Rats in the third experiment were fed a standard rodent chow (Teklad Global 18% Protein Rodent Diet, Envigo Laboratories, Indianapolis, IN). Food and water were offered to rats *ad libitum* in all three experiments.

Fecal Microbiota Experiment

Twelve seven-week old female rats were randomly assigned to each of three dietary treatment groups, low-protein (LP = 10%), moderate-protein (MP = 15%), and high-protein (HP = 20%) (n = 4 rats/diet). After one week on the experimental diets, fecal pellets were collected every three days from each rat. If a rat did not produce any feces at time of collection, collection was attempted again within 24 hours. For sample collection, a sterilized piece of foil was placed

under each rat and the rat was stimulated to defecate onto the foil by gently pressing the pellet towards the anus. Fecal pellets were placed in sterile microcentrifuge tubes using sterile forceps and then were placed in a -80°C freezer for future DNA extractions. Rats were culled after 4 weeks of sampling, using isoflurane for anesthetization and guillotine for decapitation.

Milk Microbiota Experiment

Sixteen seven-week old female rats were randomly assigned to one of two treatment groups, low-protein (LP = 10%) or high-protein (HP = 20%) (n = 8 female rats/diet; n = 4 male rats/diet were used for mating). Females were supplied with a cardboard biohut (Bio-Serv, Flemington, NJ) to encourage nest building. Females were monitored for increasing girth associated with pregnancy and moved to an isolated cage in late pregnancy.

Milk was collected from each dam at peak lactation (14 days post-partum) (3, 38, 39). At time of collection, each dam was separated from her litter and moved to a different cage with *ad lib* access with food and water, allowing milk to accumulate. When 3 hours passed, the dam was injected with 5 IU/mL of oxytocin (Osborn, Bimeda Inc, Oakbrook Terrace, IL, concentration = 20 USP per mL) intramuscularly to stimulate milk letdown. The dam was then placed with her litter for 2 – 3 minutes, allowing the pups to suckle and enhance milk letdown. Dams were anesthetized by placing them in a 4 L glass jar containing cotton balls presoaked with isoflurane. Each dam's teats were cleaned with ethyl alcohol and milk was gently expressed from 6 teats from the right side by manual palpation into a sterile capillary tube. Milk was transferred from capillary tube to a screw-top microcentrifuge tube and was flash frozen in liquid nitrogen. Rats were culled by decapitation. All samples were stored in a -80 °C freezer for future analysis. Two

females were removed from the study before milk was collected, one never became pregnant and the second cannibalized her litter.

Milk samples were thawed, vortexed, and subsampled for nutritional analysis and 16S rRNA gene sequencing (described below). Dry matter and protein content of milk was determined in triplicate. Dry matter content of milk was determined by drying 50 µg of milk to constant mass (Binder drying oven FED 115-UL, Binder Inc., Great River, NY) for 3 hours at 100°C. Protein content was determined using micro-Kjeldahl method (40, 41) as described by Hood *et al.* (42).

Common laboratory and bioinformatics methods

Fecal DNA extractions were completed using the E.Z.N.A. Stool DNA Kit (Omega Biotek, Norcross, GA) with approximately 0.2 g of fecal material and milk DNA extractions were completed using a milk microbial DNA extraction kit (Norgen Biotek Corp, Thorold, ON, Canada) with 200 µL milk. Extracted genomic DNA (gDNA) samples were nanodropped to verify DNA concentration and 40 µL replicates were sent to Molecular Research LP (Shallowater, TX) for 16S rRNA gene sequencing.

Raw data from Molecular Research Laboratories was used in a pipeline using Quantitative Insights Into Microbial Ecology (QIIME), Usearch, and Vsearch to create files for downstream and diversity analyses (43, 44). Raw data was cleaned and the mapping file was formatted. Barcodes were extracted and sequences were demultiplexed for downstream analysis. Quality filtering was done using Usearch8.1 (44) and reads were truncated to 300 bp and any reads with a quality score less than 20 were removed. Reads were dereplicated and chimeras were detected and filtered out using Vsearch1.4 (https://github.com/torognes/vsearch). Open-

reference picking in QIIME was used to pick and cluster operational taxonomic units (OTUs). Alpha- (diversity within samples) and beta-diversity (diversity between samples), Principal Coordinate Analyses (PCoA), and relative abundances of taxa were analyzed using diversity analysis in QIIME. PCoAs was used to compare groups of samples using count-based distance matrix to generate principal coordinates (PC) to show relationships between samples (45).

Culture-Dependent Milk Microorganisms Experiment

To determine if certain milk microorganisms are novel and cultivable for characterizing, culture-dependent methods were utilized to identify species that were detected with 16S rRNA sequences in the milk microbiota experiment.

Four untimed pregnant rats were individually housed as stated earlier and fed a standard rodent chow diet. Milk was collected from three rats at peak lactation as described previously. One female was removed from the study because she did not become pregnant. Milk samples were serially diluted with sterile water from 10^{-1} (undiluted) – 10^{-6}; 100 µL of each dilution was aliquoted in duplicates to each of 5 different types of agar plates. These agar plates included tryptic soy agar (TSA) with TSA as a nutrient and unselective media for maximal bacterial growth, skim milk agar (SM) with SM as a nutrient source for milk bacteria, mannitol salt agar (MSA) with MSA as a selective media for *Staphylococcus aureus*, M17 agar with M17, a cocktail of nutrients, as a selective media for *Streptococci* (46), and MRS agar containing sodium acetate and other nutrients as a selective media for *Lactobacilli* (47). All plates were incubated at 37°C aerobically and anaerobically (using anaerobic jars) for 48 hours to allow colony forming units (CFUs) to grow. Distinct CFUs were collected and plated on same media type for isolation at same incubation conditions. A CFU from pure culture plates was collected and used for

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inoculating appropriate sterile broths for growing pure culture to use for cryopreservation and DNA extraction.

Bacterial DNA extractions were completed using the E.Z.N.A. Bacterial DNA Kit (Omega Biotek, Norcross, GA) with approximately 1.5 mL of culture broth. Extracted gDNA resulted in 100 µL replicates that were stored at -20 °C until they were used as templates for PCR for amplifying DNA for sequencing. The two primers selected for PCR amplification of bacterial 16S rRNA gene were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') (48). Reaction mixtures (50 µL) contained 25 µL of EconoTaq PLUS Green 2x Master Mix (Lucigen Corporation, Middleton, WI), 0.5 µL of each primer (final concentration: 20 µM), 5 µL of DNA, and 19 µL of water were used. PCR conditions were an initial denaturation temperature step of 95°C for 3 minutes, 30 cycles (95°C and 50°C each for 1 minute and 72°C for 2 minutes), and extension temperature of 72°C for an additional 5 minutes after the 30 cycles. A total of 5 µL of PCR-amplified DNA was analyzed on a 1% (wt/vol) agarose gel with ethidium bromide staining. gDNA quality was assessed using agarose gel electrophoresis and all samples were purified using the E.Z.N.A. Cycle Pure Kit (Omega Biotek, Norcross, GA). Purified DNA samples were sequenced at Auburn University's Genomics and Sequencing Lab using 6 µL reaction mixtures consisting of 1 µL of 7nM 27F primer and 5 µL of DNA. Low quality reads were trimmed and sequences were aligned using MEGA version 7 (49) and queried using BLASTn against the GenBank database of nucleotide (nr/nt) sequences (50, 51).

Statistical Analyses

Statistical analyses were done using R version 3.2.2 (52) and MiniTab version 17.2.1 (53). Comparisons of fecal bacterial phyla and genera relative abundances between treatment groups were completed using repeated measures to account for autocorrelation. Monte-Carlo permutations for all were done 999 times. Linear regression was used to compare average body mass of litters between groups. Analysis of Similarities (ANOSIM) was used to compare if microbial communities are dissimilar between treatment groups; identical communities are given an R statistic close to 0 and dissimilar communities are given a value close to 1. Kruskal-Wallis tests were used to compare relative abundance of microbial taxa in milk between dietary treatment groups. Statistical significance was established at $p < 0.05$.

RESULTS

Fecal microbiota community diversity affected by diet

A total of 184,250 reads were produced from 46 fecal samples by 16S rRNA sequencing rat fecal DNA after quality-filtering and a mean sample depth of 4005 reads with a standard deviation of 539 reads from fecal samples. 3120 randomly selected sequences were evaluated from all samples to provide estimates for alpha diversity. Alpha diversity and phylogenetic distances of microorganisms in feces were highest for rats consuming LP diet (Fig. 1A, p=0.002). Rats displayed no difference in either metric when consuming MP or HP diet. A significant difference was observed for Shannon-Wiener's Index (54, 55), observed OTUs, and predicted OTUs (56) when LP was compared to MP- and HP-fed rats (Fig. 1B-1D) (Table 2). No temporal variation was observed within treatment groups in the alpha diversity of fecal samples..

Diet had an effect on bacterial communities in rat feces when measured by Weighted UniFrac distances using ANOSIM (p=0.001; R=0.40). Weighted UniFrac is a quantitative

measure that accounts for differences in communities due to changes in relative taxon abundance (57). Samples are clustered by diet and differences in communities can be seen in PCoA plots that captured 55% total variation in two primary principal coordinates (Fig. 2).

Fecal Actinobacteria and Bacteroidetes were higher in LP-fed rats than MP- or HP-fed rats (Table 2). Relative abundance of Firmicutes increased as dietary protein intake increased (Table 2). Microbial communities in rat feces were stable with *Bacteroidaceae*, *Rikenellaceae*, *S24-7 Clostridiales*, *Lachnospiraceae*, and *Ruminococcaceae* comprising a majority of the community (Table 1). *Bifidobacteriaceae*, *Rikenellaceae*, *S24-7*, *Clostridiales*, and *Ruminococcaceae* are statistically higher in abundance in LP-fed rats than in MP- and HP-fed rats (Table 1). Within Firmicutes, a trend was observed with *Lactobacillus* abundance being higher in HP fecal samples than LP and MP samples (p=0.06). There was no difference in relative abundance over time.

Milk composition and pup condition

No differences were observed for dry matter (LP=3.94±0.35%, HP=4.65±0.19%, p=0.18) or crude protein content (LP=20.7±1.30%, HP=22.2±0.71%, p=0.40) between LP and HP milk samples. Pups from each litter were weighed to determine if a mother's diet and her litter size affected average pup mass at 14 days of age. Body mass of 14-day-old pups (corrected for litter size) of LP-fed dams averaged 14.1±1.7g lower than 14-day-old pups of HP-fed dams (p=4.65e^{-09}; r^2=0.96; Fig. 3).

Dietary effect on milk microbial diversity

A total of 27,182 reads was produced by 16S rRNA sequencing after quality-filtering and a mean sample depth of 1941 reads with a standard deviation of 703 reads from milk samples. I observed differences in alpha diversity with rats consuming LP having lower phylogenetic distance compared to rats consuming HP diet (Fig. 4; p=0.01; r^2=0.41). Shannon-Wiener Index, predicted OTUs, or observed OTUs did not differ between groups.

Diet affected bacterial communities in rat milk when measured by Unweighted UniFrac distances using ANOSIM (p=0.002; *R*=0.41). Unweighted UniFrac is a qualitative measure that detects differences in presence or absence of bacterial lineages in different communities (57). PCoA of Unweighted UniFrac distances resulted in greatest separation between HP and LP diets along PC3 versus PC2 with 28.7% variation explained and 4 LP samples being clustered along PC3 (Fig. 5).

Milk bacterial assemblages were dominated by the following phyla: Actinobacteria, Firmicutes, and Proteobacteria. *Micrococcaceae* made up most of Actinobacteria with similar percentages between both treatment groups, although *Bifidobacteriaceae* was statistically different between HP and LP milk samples (p=0.307; p=0.015, respectively; Table 3). Within Firmicutes, *Staphylococcaceae* was different and slightly increased relative abundance in LP milk samples (p=0.041), *Lactobacillaceae* and *Clostridiales* had increased abundance in HP samples (p=0.022; p=0.0022, respectively; Table 3). *Lachnospiraceae* had increased relative abundance in HP samples (p=0.015). Proteobacteria families *Rhizobiaceae*, *Pasteurellaceae*, and *Xanthomonadaceae* dominated in milk samples for both treatment groups (Table 3).

Enumeration and 16S rRNA sequences of milk microorganisms

Bacterial CFUs in all samples ranged from 0 to 10^6 CFU/mL. Aerobically incubated plates had higher average CFU counts than anaerobically incubated plates. TSA and M17 plates showed greatest numbers of CFUs compared to SM, MSA, and MRS. MRS had lowest CFU counts and 10^{-3} plates did have not yield a single CFU. Thirty-four isolates were collected from the five different media plates from milk collected from three rats. Sequencing of the 16S rRNA gene sequences using the 27F primer and comparison of the trimmed sequences against the GenBank nr/nt database indicated that 10 isolates had a top BLAST as *Proteus mirabilis* and 3 isolates had a top BLAST hit as *Enterococcus faecalis*.

DISCUSSION

The results of this investigation confirm that dietary protein intake affects gastrointestinal tract microbial diversity and suggest that a mother's diet impacts microbial communities found in her milk. This investigation is also novel because, to my knowledge, there are no studies that have explored rat milk microbial diversity and cultured rat milk microorganisms. Many recent studies have explored the effects of high-carbohydrate or high-fat diets on gut microbial diversity to understand why such diets may contribute to metabolic diseases (58). Prior research on the impacts of high-protein diets on gut microbiota have shown an increase in short-chain fatty acid-producing bacteria, which can affect circulating hormones, and reduced fecal microbial diversity (59-61). Interestingly, while gut microorganisms of LP females displayed greater diversity of microbial species, the opposite was true for microbial diversity in milk.

Fecal microbiota affected by dietary protein intake

Alpha diversity in LP- and MP-fed rats had higher species richness and diversity based on Shannon-Wiener Index values indicating that rats in these groups had higher number of duplicate bacterial species when compared to HP-fed rat fecal samples. Phylogenetic distance was lower with higher protein intake in diets suggesting that rats consuming HP diet have bacteria that are more closely related to each other than bacteria in LP fecal samples. Reduction in diversity implies gut microbiota may become increasingly specialized for amino acid fermentation and increase production of short-chain fatty acids when greater protein is available in an animal's diet (12).

Results of PCoA showed relative protein intake can affect microbial diversity in feces. While MP and HP samples clustered together, LP fecal samples displayed clear variation in relative abundance of taxa. MP- and HP-fed rats had a comparable Bacteroidetes:Firmicutes ratio while LP-fed rats had a higher Bacteroidetes:Firmicutes ratio. Clinton (62) showed that increasing the protein content in the diet can increase protein breakdown and ammonia production which is a harmful secondary metabolite produced by colonic bacteria (63). It is probable that higher protein intake could be causing a reduction in microbial diversity in HP-fed rat feces and shifting dominance towards Firmicutes. Predominant bacterial phyla found in feces of rats in this study were similar to previous studies done in kittens and dogs; particularly with regard to the relative ratio of Bacteroidetes to Firmicutes (64, 65). The Bacteroidetes:Firmicutes ratio in a similar study done with rats reported that rats consuming a normal protein diet had a higher Bacteroidetes:Firmicutes ratio relative to rats consuming a high protein diet (66). A study comparing dietary impact on fecal microbiota in children from Europe and Africa found that children from Africa had lower protein intake, higher fiber content, and lower caloric intake had

a higher Bacteroidetes:Firmicutes ratio and children from Europe showed opposite trends with protein and fiber intake and Bacteroidetes:Firmicutes ratio (67).

While the relative difference was small, the abundance of *Bifidobacterium* was greater in feces of LP-fed rats than MP- or HP-fed rats. *Bifidobacterium* is a well known genus within Actinobacteria that are, common in animal gastrointestinal tracts. Some species of *Bifidobacterium* have been used as probiotics due to their antimicrobial activity against pathogenic bacteria (68). *Bifidobacterium* utilize carbohydrates as a nutrient source for fermentation (69) and thus it is not surprising that relative abundance of this microorganism was greater in the feces of rats on the LP diet, which is higher in carbohydrates than MP and HP diets. *Clostridiales* and *Ruminococcaceae* abundances are higher in MP- and HP-fed rat feces than the LP-fed rat feces, likely due to increased dietary protein in hindgut that is available to be used by both taxa to produce short-chain fatty acids by protein fermentation (70, 71). However, metabolic activities of protein-fermenting microorganisms causes production of nitrogenous metabolites like ammonia, which could inhibit growth of *Bifidobacterium* in MP- and HP-fed rats, as proposed by Hooda *et al.* (59). Hooda *et al.* (59) found the same effect of high-protein/low-carbohydrate diet on *Bifidobacteria* in kittens. Presence of *Bifidobacterium* in rat feces suggests that their gastrointestinal tracts were not under microbial dysbiosis.

Frese *et al.* (72) reported how that pigs shifting from mothers milk to swine chow at weaning had abrupt taxonomic shift in gut microbiome. A caveat of my study is a lack of day zero results of collecting feces from when the rats were switched to their experimental diets that would have been valuable for determining core microbiome of rat feces before applying dietary treatment. Given a week to acclimate to a new diet and excluding dietary effects, fecal

microbiota did not change, suggesting gastrointestinal microbial community changed and reached equilibrium in less than one week.

A mother's dietary protein intake impacts offspring development

A mother undergoing lactation needs to maintain a higher dietary protein intake to ensure that her milk quality is sufficient to support offspring development. Dietary protein intake is strongly correlated to a mother's litter mass (73) and an inadequate diet that does not supply enough protein necessary for lactogenesis will consequently result in malnourished offspring. Litter size also affects offspring development due to constraints on total volume and quality of milk a mother can produce, resulting in reduced body mass in pups born into the largest litters (74). In this investigation, both diet and litter size impacted average mass of the litter. No differences in dry matter or protein content of milk was observed between females fed LP and HP diets, suggesting differences in milk microbiota, rather than milk composition, could contribute to differences in pup growth. However, dietary protein during lactation has been shown to impact relative milk yield (75-77) and dietary protein content has also been shown to be positively correlated with relative protein content of a mother's milk (75, 78, 79); therefore, it is possible that small differences in milk composition occurred between groups were not detected due to a limited sample size. Although it is possible that milk microbiota played a role in growth and development of young, this study does not have sufficient data to suggest that differences in milk microbiota were responsible for differences in observed pup development. However, this finding does highlight the need for further investigation.

Milk microbiota affected by dietary protein intake

Compared to fecal microbiota, alpha diversity and phylogenetic distance of milk microbiota of LP-fed rats were lower than HP-fed rats. Bacteroidetes were almost nonexistent in milk while they are common in LP-fed rat feces. Increased Bacteroidetes abundance is commonly associated with lean phenotypes (80). However, their low presence in milk suggests that there is limited transfer of gut Bacteroidetes to milk or they do not persist after transport. Ward *et al.* (81) also indicated that Bacteroidetes were commonly seen in mother and infant feces, but comprised less than 1 percent of the milk metagenome. No differences in relative abundance of *Rothia* being affected by diet could mean that it can be originating from dam teats or offspring mouths and could enter the milk during suckling because *Rothia* is a common oral and skin bacterial genus associated with dental caries.

Presence of *Rhizobiaceae* in milk samples consisted of genus *Agrobacterium*. This genera includes, but is not limited to, common plant pathogens that cause crown-gall tumors in plants (82). Despite no differences in relative abundance for *Agrobacterium* between treatments, comprising about 15% of milk bacterial sequences is an anomaly. McInnis *et al.* (83) reported presence of *Agrobacterium* and other Proteobacteria in raw goat milk, but their relevance to health and milk quality is still unknown. A few *Agrobacterium spp.* have been found in termite gut (82) and in Arctic charr small intestines that were fed high levels of carbohydrates and could contribute to nutritional processes (84). I believe *Agrobacterium* was found in rat milk associated with uptake from their plant-based diet or from the pine contact bedding in their boxes. This could be further investigated by evaluating microbial diversity of the diets and bedding. It is possible that *Agrobacterium* is transferred to offspring via milk may contribute to the degradation of cellulose or other complex carbohydrates in hindgut.

Staphylococcus displayed increased relative abundance in LP milk which could mean that higher levels of potential pathogens could be transferred to offspring and potentially reduce growth performance. Different species of *Staphylococcus* are found in milk, but *S. aureus* is commonly associated with mastitis. Commensal *Staphylococci* have been found in human breast milk along with pathogenic strains (85). *S. aureus* is known to compete with lactic acid bacteria like *Lactobacillus* for nutrients and can metabolize different carbon sources, including lactose, which can facilitate its growth in milk (86). Dietary protein intake may play a role with inhibiting pathogenic strains of *Staphylococcus* from being transferred to offspring by enriching growth of lactic acid bacteria to inhibit *Staphylococci* growth in milk.

A trend suggested that *Lactobacillus* abundance was greater from rats consuming the high-protein diet and a similar pattern was also observed in milk. This pattern suggests that the increase in fecal *Lactobacillus* could be responsible for the increase described in milk. Rat dams consuming a high-protein diet have milk containing a higher percentage of *Lactobacillus* is a common to the fecal and milk microbiome across species and also produces bacteriocins to inhibit growth of pathogens in milk (87).

Clostridia spp. are potentially health promoting microorganisms found in higher abundance in HP than LP milk samples similar to *Lactobacillus* (9). *Clostridia* is important with activating immunologic functions in neonates and is utilizing amino acids and producing short-chain fatty acids with other proteolytic milk bacteria. *Clostridia* producing butyrate can also lead to downregulation of virulent gene expression (88). However, not all *Clostridia* are mutualistic towards their host. *Clostridium difficile* is associated with irritable bowel syndrome and diarrhea and *Clostridium perfringens* is an opportunistic pathogen that causes tissue destruction in hosts (89-91). It is possible that HP milk samples that *Lactobacillus* and *Clostridia* could be inhibiting

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growth of *Staphylococcus* and reducing potential pathogen transferal to offspring as a positive maternal effect on offspring development.

Cultivable rat milk microorganisms

Bacteria in rat milk are capable of being grown in culture. While a lot of research has been done on gut microorganisms in rats, there is much to learn about how gut microorganisms are related to ones found in milk. Identification of *P. mirabilis* is unusual due to no presence of it from the fecal microbiota and it is found with less than 1% relative abundance in milk microbiota 16S rRNA data. *P. mirabilis* is associated with being part of human gut microbiota and has the ability to ferment sugars except lactose (92). It is reasonable that presence of *P. mirabilis* for most isolates was due to contamination, because isolates were chosen by CFU morphology and pigmentation to try to identify different cultured microorganisms possible. However, no data on rat milk microorganisms could account for a novel microorganism that is related to *P. mirabilis* that could be easily cultured from milk. *P. mirabilis* is able to change its morphology and that could explain why it was identified from many of the isolates grown on different media (93). *E. faecalis* is also unusual because it is found in the milk microbiota 16S rRNA data with less than 1% relative abundance like *P. mirabilis,* but no presence in feces. Certain strains of *E. faecalis* are associated to be pathogenic (94, 95), but a strain of *E. faecalis* from whey cultures has been shown to produce a bacteriocin to inhibit growth of *Listeria monocytogenes* (96). Further investigation is needed using tests to verify presence of *P. mirabilis* and *E. faecalis* in culture. Culturing bacteria from rat milk may provide an understanding of the functions of specific microorganisms in rat milk and give us insights into how milk microorganisms may contribute to offspring development.

Conclusions

My thesis highlights how dietary protein intake can shift microbial diversity in rat feces and milk. I have shown that high-protein diets can decrease microbial diversity in feces from non-lactating rats is similar to previous work done with growing kittens (59). Higher protein intake appeared to have a greater abundance of potential health promoting bacteria found in milk, such as *Lactobacillus* and *Clostridia*, while potential pathogens abundance like *Staphylococcus* are decreased. Future work with milk will involve examining possible interactions between *Lactobacillus* and *Staphylococcus* can further highlight the importance of diet with offspring development through selection of beneficial bacteria for offspring gut microbiota.

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Table 1. Macronutrient profile of the custom diets used in this experiment (TestDiet, Purina Animal Nutrition, LLC), including low-protein (LP), moderate-protein (MP), high-protein (HP) and 2018 Teklad Global 18% Protein Rodent Diet (Envigo Laboratories) standard rodent diets fed to Sprague-Dawley rats.

Components[#]	Diets			
	LP	MP	HP	Standard
Dry matter (DM, % as is)	90.5	90.6	90.5	92.5
Crude protein (% DM)	10.3	15.1	20.9	18.6
Fat (acid hydrolysis, % DM)	5.70	5.80	5.50	6.20
Ash (% DM)	3.90	4.70	6.00	5.30
Crude fiber (% DM)	4.40	4.50	4.80	3.50
Carbohydrates (% DM)	66.2	60.5	53.3	44.2
Metabolizable energy (kcal/g)	3.53	3.49	3.42	3.10

[#]Micronutrients were similar between all diets.

Table 2. Predominant bacterial phyla and genera (percentage of readings) in rat feces fed a low-protein (LP), moderate-protein (MP), or high-protein (HP) diet. (Mean values with their standard error mean, n = 4) P-values were generated using repeated-measures. Asterisks indicate unidentified orders and ampersands indicate unidentified families. Significance was established at p < 0.05.

Phylum	Genus	Diets			P-Value		
		LP	MP	HP	LP vs MP	LP vs HP	MP vs HP
Actinobacteria		2.72±0.45%	0.29±0.09%	0.26±0.04%	**0.000**	**0.000**	0.316
	Bifidobacterium	1.71±0.28%	0.03±0.01%	0.03±0.00%	**0.000**	**0.000**	0.880
	Coriobacteriaceae&	0.54±0.15%	0.03±0.01%	0.02±0.00%	**0.001**	**0.001**	0.950
Bacteroidetes		32.2±1.61%	26.2±1.48%	23.7±1.07%	**0.019**	**0.002**	0.305
	Bacteroides	6.23±0.64%	5.99±0.85%	5.51±0.59%	0.840	0.489	0.625
	Rikenellaceae&	5.80±0.28%	5.37±0.26%	3.40±0.33%	0.258	**0.000**	**0.000**
	*S24-7**	18.7±0.97%	13.5±0.70%	13.7±0.60%	**0.001**	**0.002**	0.850
Firmicutes		61.5±1.83%	70.3±1.49%	72.6±1.12%	**0.001**	**0.000**	0.360
	Lactobacillus	2.45±0.43%	2.29±0.38%	3.44±0.45%	0.695	0.064	**0.030**
	Turicibacter	2.02±0.22%	0.43±0.15%	0.10±0.02%	**0.000**	**0.000**	0.155
	*Clostridiales**	22.6±1.16%	30.7±1.31%	26.7±1.63%	**0.000**	**0.042**	**0.053**
	Christensenellaceae&	0.21±0.03%	0.12±0.03%	0.74±0.07%	**0.044**	**0.000**	**0.000**
	Clostridiaceae&	3.01±0.37%	0.72±0.23%	4.15±0.17%	**0.000**	**0.000**	0.301
	Blautia	0.13±0.03%	0.11±0.03%	0.70±0.18%	0.788	**0.000**	**0.000**
	Coprococcus	1.59±0.20%	2.43±0.31%	1.92±0.25%	**0.002**	0.216	**0.044**
	Lachnospira	0.10±0.05%	0.00±0.00%	0.24±0.12%	0.299	0.168	**0.021**
	Peptococcaceae&	0.15±0.02%	0.26±0.02%	0.16±0.03%	**0.001**	0.999	**0.001**
	RC4-4	0.41±0.05%	0.25±0.05%	0.54±0.11%	0.134	0.305	**0.015**
	Ruminococcaceae&	8.28±0.55%	9.06±0.61%	11.6±0.95%	0.595	**0.012**	**0.034**
	Oscillospira	6.02±0.55%	9.28±0.71%	7.28±0.73%	**0.001**	0.187	**0.039**
	Ruminococcus	6.95±0.38%	6.99±0.41%	7.79±0.61%	0.871	0.166	0.213
Proteobacteria		2.03±0.25%	1.98±0.20%	2.23±0.17%	0.187	**0.041**	0.474
	Bilophila	0.05±0.02%	0.39±0.07%	0.24±0.06%	**0.000**	**0.025**	**0.050**
	Acinetobacter	0.71±0.25%	0.45±0.12%	0.30±0.07%	0.187	**0.041**	0.474

Table 3. Predominant bacterial phyla, families, and genera (percentage of readings) in milk of rats fed a low-protein (LP) or high-protein (HP) diet. (Mean values with their standard error mean, n = 7) P-value were generated using Kruskal-Wallis test. Asterisks indicate unidentified orders and ampersands indicate an unidentified families. Significance was established at $p < 0.05$.

| Phylum | Family | Genus | Diets | | P-Value |
			LP	HP	LP vs HP
Actinobacteria			26.3±4.90%	19.8±7.93%	0.306
	Micrococcaceae	*Rothia*	23.7±5.16%	18.0±7.90%	0.307
	Bifidobacteriaceae	*Bifidobacterium*	1.00±0.42%	0.02±0.00%	**0.013**
Firmicutes			35.6±6.20%	41.5±9.23%	0.702
	Planococcaceae[&]	Unidentified	2.93±1.57%	0.95±0.04%	0.199
	Staphylococcaceae	*Staphylococcus*	12.5±6.04%	4.27±1.27%	**0.041**
	Lactobacillaceae	*Lactobacillus*	1.51±0.28%	10.0±3.76%	**0.022**
	Streptococcaceae	*Streptococcus*	15.0±3.81%	8.40±1.49%	0.125
	*Clostridiales**	Unidentified	0.08±0.04%	4.60±2.43%	**0.002**
	Lachnospiraceae[&]	Unidentified	0.14±0.07%	4.93±2.37%	**0.013**
	Lachnospiraceae	*Blautia*	0.07±0.07%	1.37±0.51%	**0.004**
	Ruminococcaceae[&]	Unidentified	0.19±0.21%	2.38±1.90%	**0.030**
	Ruminococcaceae	*Ruminococcus*	0.15±0.11%	1.10±0.54%	0.111
Proteobacteria			37.2±8.22%	36.8±8.65%	0.898
	Rhizobiaceae	*Agrobacterium*	14.1±5.20%	15.6±5.91%	1.000
	Comamonadaceae	*Delftia*	1.53±0.29%	1.78±0.45%	0.898
	Pasteurellaceae	*Haemophilus*	1.26±0.36%	2.12±0.52%	0.250
	Pasteurellaceae	*Mannheimia*	5.78±1.86%	2.96±1.38%	**0.041**
	Xanthomonadaceae[&]	Unidentified	4.37±1.50%	3.70±1.05%	0.609
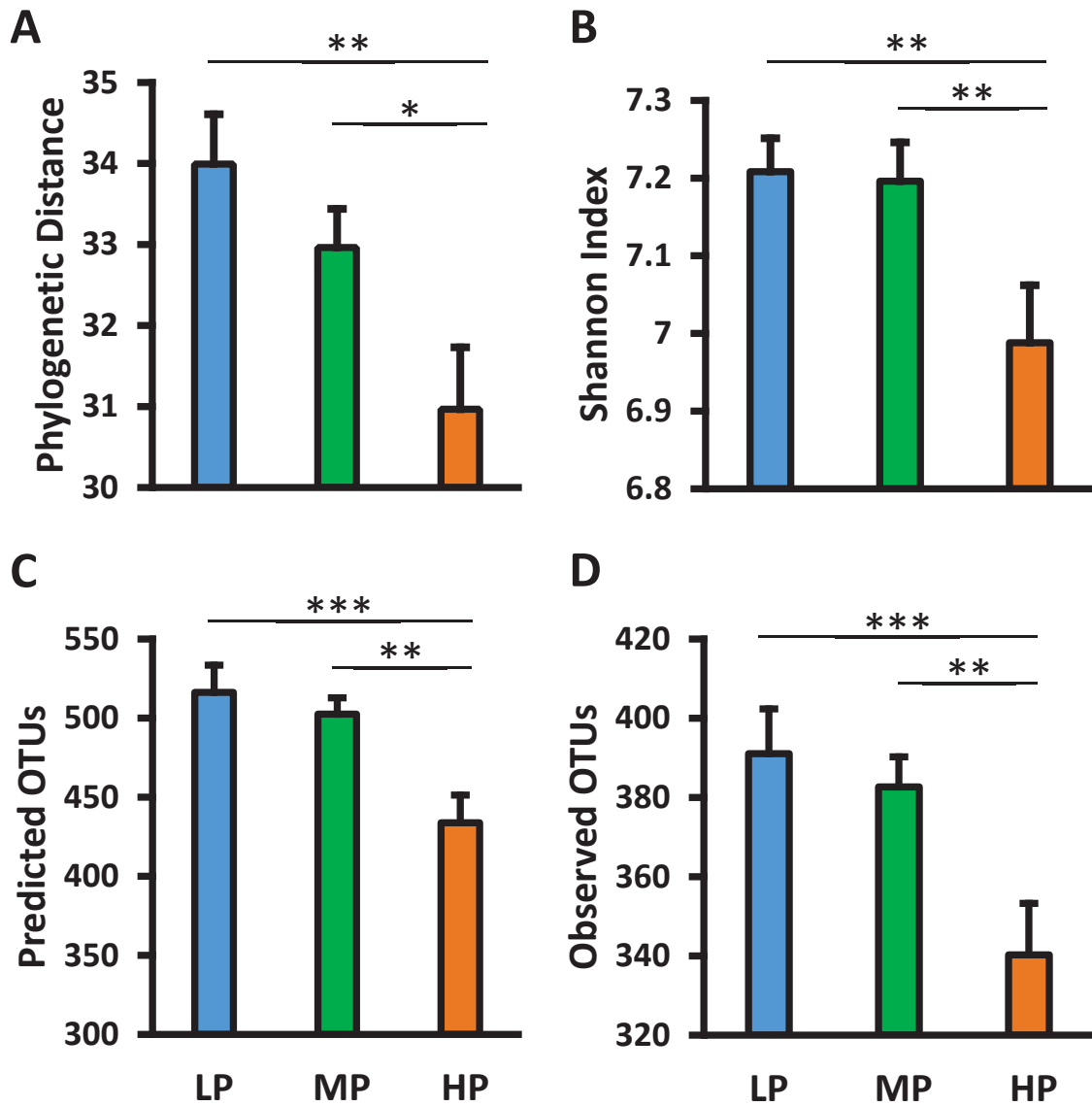
Figure 1. Rat fecal microbiota alpha diversity compared between diets using phylogenetic distance (**A**), Shannon-Wiener Index (**B**), predicted OTUs (**C**), and observed OTUs (**D**). Bar graphs show means and standard error bars. Asterisks indicate statistical differences dietary treatments (repeated measures, * $p < 0.05$, ** $p \leq 0.01$, and *** $p < 0.0001$).
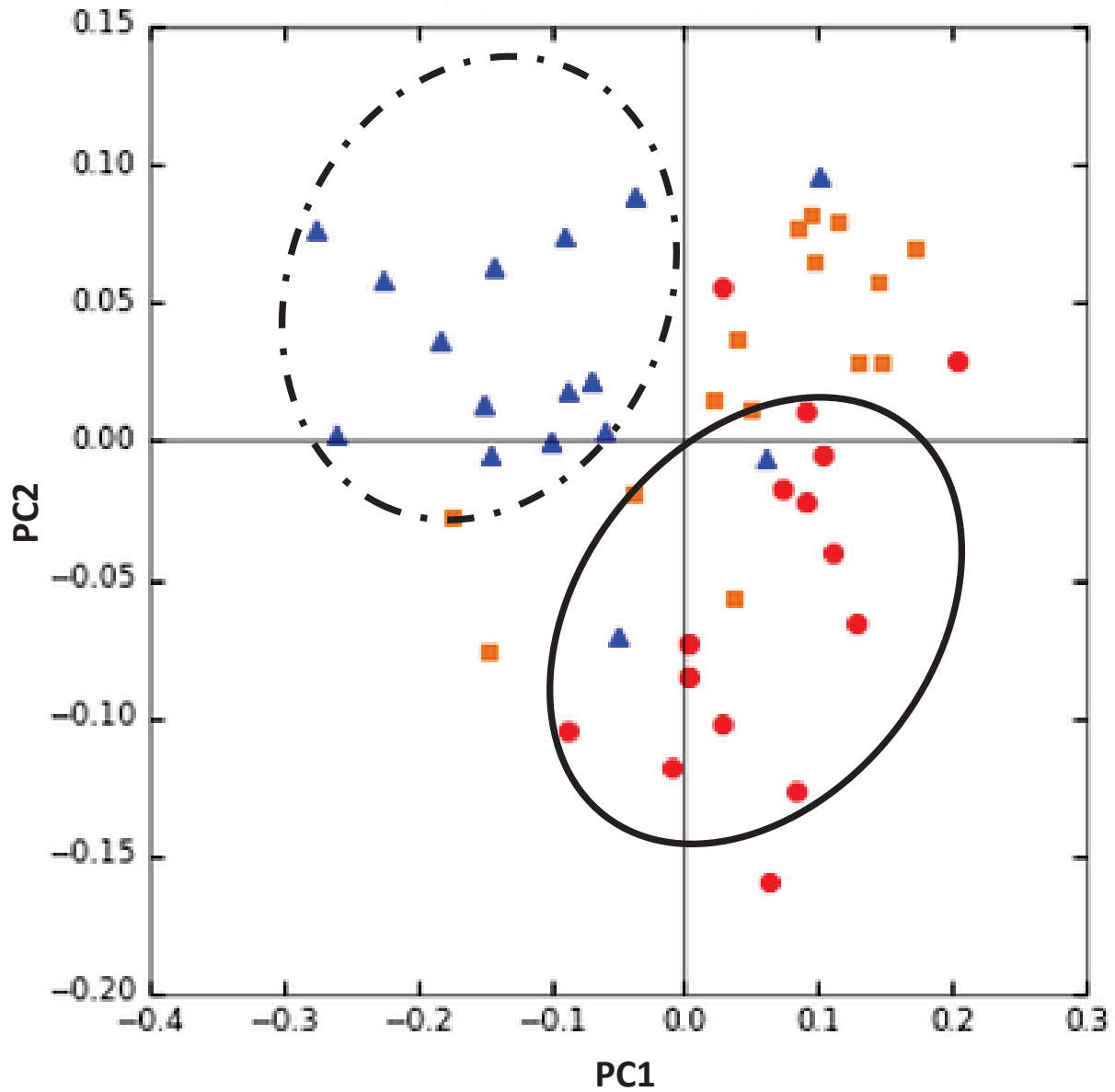
Figure 2. Principal coordinate analysis plot of rat fecal microbiota by diet (LP, blue triangles; MP, orange squares; HP red circles). Plot was generated using Weighted UniFrac distances. PC1 and PC2 respectively explain 42.61% and 12.28% variation.
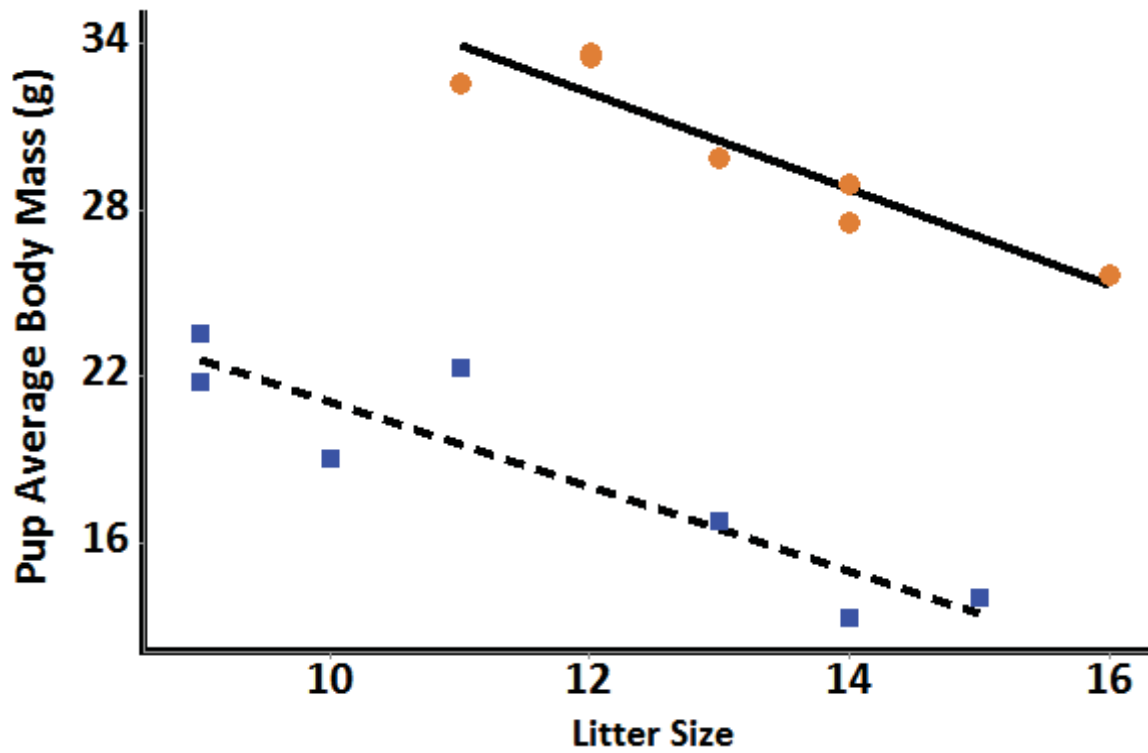
Figure 3. Average body mass of rat pups in a litter versus litter size when grouped by dietary treatment (LP litters are blue squares, HP litters are orange circles).
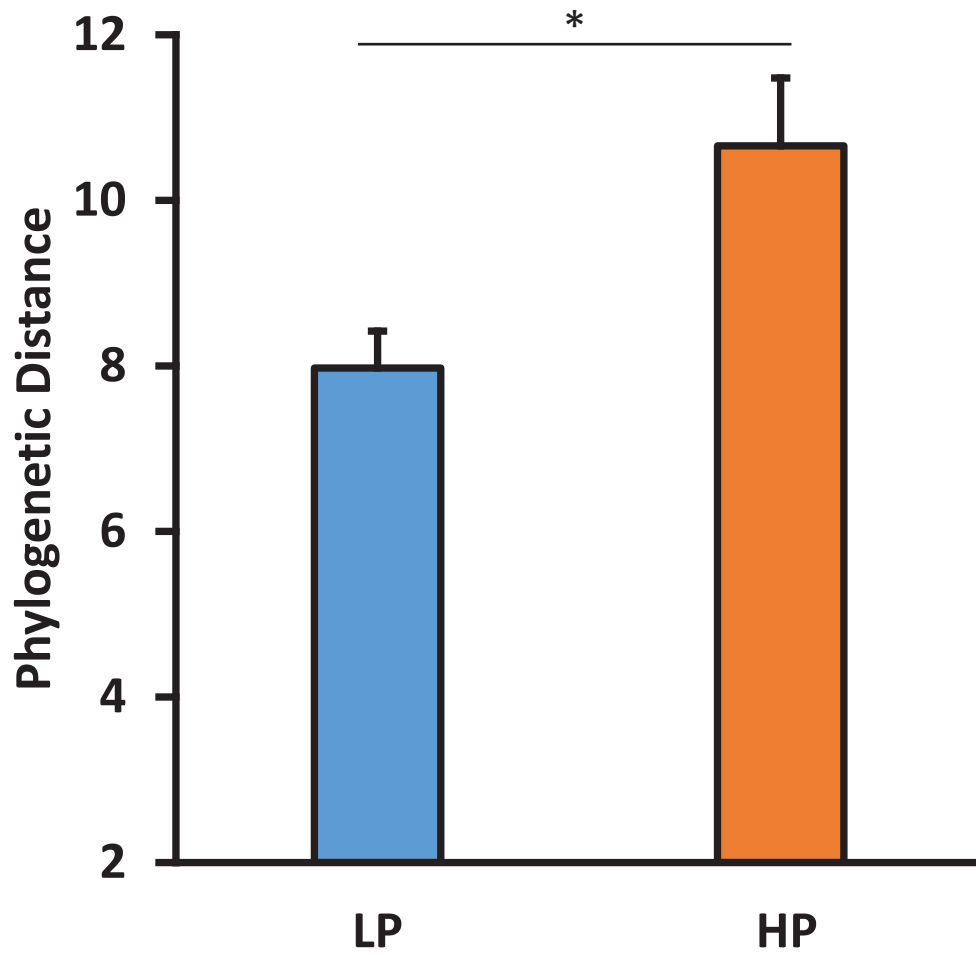
Figure 4. Rat milk microbiota alpha diversity compared between diets using phylogenetic distance. Asterisk indicates statistical difference (linear regression of all data points, * $p < 0.05$).
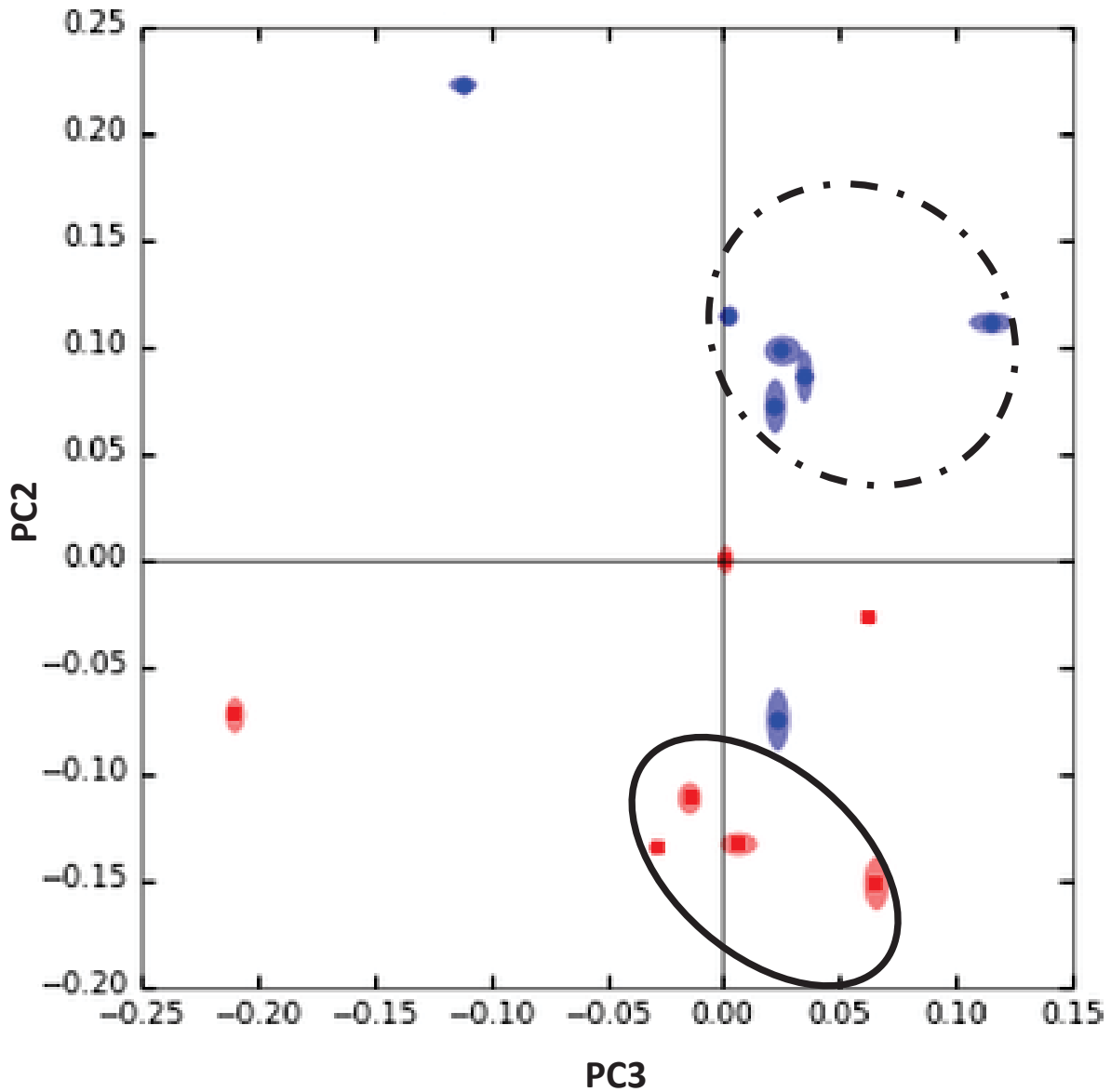
Figure 5. Principal coordinate analysis plot of rat milk microbiota by diet (LP, blue circles; HP, red squares; an ellipsoid surrounding a sample indicates confidence interval for that sample). Plot was generated using Unweighted UniFrac distances. PC3 and PC2 respectively explain 9.33% and 19.34% variation.