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Age-related changes in the marmoset gut microbiome

Kelly R. Reveles[1,2], **Shrina Patel**[1,2], **Larry Forney**[3], and **Corinna N. Ross**[4,5,6]

[1.] College of Pharmacy, The University of Texas at Austin, Austin, TX, USA

[2.] Pharmacotherapy Education & Research Center, University of Texas Health San Antonio, San Antonio, TX, USA

[3.] Department of Biological Sciences, University of Idaho, Moscow, ID, USA

[4.] Department of Science and Mathematics, Texas A&M University San Antonio, San Antonio, TX, USA

[5.] Barshop Institute for Longevity and Aging Research, University of Texas Health San Antonio, San Antonio, TX, USA

[6.] Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio, TX

Abstract

The gut microbiome is known to play a significant role in human health but its role in aging remains unclear. The objective of this study was to compare the gut microbiome composition between young adult and geriatric nonhuman primates (marmosets) as a model of human health and disease. Stool samples were collected from geriatric (8+ years) and young adult males (2–5 years). Stool 16s rRNA V4 sequences were amplified and sequenced on the Illumina MiSeq platform. Sequences were clustered into operational taxonomic units and classified via mothur's Bayesian classifier referenced against the Greengenes database. A total of 10 young adult and 10 geriatric marmosets were included. Geriatric marmosets had a lower mean Shannon diversity compared to young marmosets (3.15 vs. 3.46; p=0.0191). Geriatric marmosets had a significantly higher mean abundance of Proteobacteria (0.22 vs. 0.09; p=0.0233) and lower abundance of Firmicutes (0.15 vs. 0.19; p=0.0032) compared to young marmosets. Geriatric marmosets had a significantly higher abundance of Succinivibrionaceae (0.16 vs. 0.01; p=0.0191) and lower abundance of Porphyromonadaceae (0.07 vs. 0.11; p=0.0494). In summary, geriatric marmosets had significantly altered microbiome diversity and composition compared to young adult marmosets. Further studies are needed to test microbiome-targeted therapies to improve healthspan and lifespan.

Keywords

microbiome; gut microbiota; dysbiosis; aging; geriatrics; marmoset

Contact Information: Corinna Ross, PhD, Associate Professor of Biology, Texas A&M University San Antonio, One University Way, San Antonio, TX 78224, Phone: 210-784-2227, Corinna.Ross@tamusa.edu.

Conflicts of Interest

The authors report no competing interests related to the content of this manuscript.

Introduction

An individual's health and wellbeing were once thought to be solely associated with their own genes and environment; however, it has become clearer that the health of an individual is also influenced by a broader metagenome phenotype as a result of our microbiome. Trillions of microorganisms live commensally within the gut and serve several important physiological functions, including protection against pathogens, immune response regulation, nutrient and energy extraction, and hormone biosynthesis (Belkaid & Hand, 2014; Lynch & Pedersen, 2016). Intestinal microbial diversity in nonhuman primates has been found to be tightly linked to the host phylogeny (Yildirim et al., 2010), to the social group that the individuals are interacting with (Amato et al., 2017), and to the components of their dietary intake (Hicks et al., 2018).

It is not surprising then that dysbiosis (i.e., disturbance or change in the microbiota) has been associated with increased inflammation and altered physiological homeostasis (Cattaneo et al., 2017). Evaluations of nonhuman primates in captive settings have revealed significant loss of microbial diversity for animals maintained in captivity when compared to the same species in wild, or semi-captive settings (Clayton, Gomez, et al., 2018; Clayton et al., 2016). This shift in diversity appears to be associated with changes in dietary components. Red-shanked doucs in captivity have been found to have increased dysbiosis associated with significantly reduced Firmicutes: Bacterioidetes ratios associated with increased risk of wasting syndrome, gastrointestinal disease, and mortality (Clayton, Al-Ghalith, et al., 2018). An evaluation of a macaque model of obesity revealed that the dam's dietary intake and not her status as obese or lean significantly altered the infant's developing intestinal microbiome (Ma et al., 2014). In humans, dysbiosis of intestinal microbiota has been associated with obesity, type 2 diabetes, Alzheimer's disease, cardiovascular disease, and frailty (Barlow, Yu, & Mathur, 2015; Jackson et al., 2016; Komaroff, 2017). In many cases, it remains unclear whether changes in the microbiome are a result of the disease or whether alterations in the microbiome leave the host vulnerable for physiological changes contributing to the disease state.

Human studies have also noted a significant decline in microbiome diversity and composition with age, (Yatsunenko et al., 2012) which might contribute to functional decline and age-related disease status; however, changes in medication exposure, physical activity, and diet with age could also significantly contribute to dysbiosis. A shift in the microbiota toward a Bacteriodetes dominated population is associated with frailer older humans (Jeffery, Lynch, & O'Toole, 2016). Centenarians have been found to have significantly increased populations of pathobionts including Bacteriodetes and Proteobacteria and shifts in Firmicutes diversity (Rampelli et al., 2013). However, these shifts in the gut microbial community are also associated with changes in the diet to low fiber diets with less macronutrient diversity typical of nursing home meals. Therefore, it is unclear whether the microbial shift is a result a dietary change, or whether the microbial shift is associated with the aging process and leads to inflammation and disease. These confounders make the study of the microbiome in aging humans particularly challenging.

Evaluating the interactions between the microbiome and phenotypic aging in a nonhuman primate model offers the advantage of being able to more definitively control environment, diet, and exposure to medication. The marmoset offers a unique nonhuman primate in which to evaluate hypotheses associated with both aging and healthspan. Marmosets have a short lifespan when compared to other primates; they reach sexual maturity between 12–18 months and full adult weight by 24 months (Tardif, Mansfield, Ratnam, Ross, & Ziegler, 2011). They are considered young adults from 2–5 years of age and are considered geriatric over the age of 8 years. Marmosets are relatively inexpensive to house, and they are not zoonotic hosts making them easier and safer to handle for experimentation than many other nonhuman primate biomedical models. Furthermore, the recent expansion of phenotypic assessments of aging have found that marmosets display many aging characteristics that mimic human aging, including cardiovascular changes, inflammatory disease, and cognitive decline (Ross, Davis, Dobek, & Tardif, 2012) (other manuscripts in this special issue). Therefore, our study objective was to compare the gut microbial diversity and composition between young adult and geriatric marmosets. We hypothesized that geriatric marmosets would have significantly less diverse and compositionally different microbiomes than young adult marmosets independent of differences in diet or housing.

Methods

Study Design

This was a cross-sectional study of marmosets housed at the Barshop Institute for Longevity and Aging Studies in San Antonio, TX in August 2017. The study was approved by the Institutional Animal Care and Use Committee at UT Health San Antonio, and follows guidelines set forth by the American Society of Primatologists. Marmosets housed at the Barshop Institute are primarily a breeding colony of animals maintained in barrier (Ross et al., 2017) or semi-barrier conditions (isolated from other nonhuman primates) specifically for aging related research with husbandry protocols following those outlined in (Layne & Power, 2003). The marmosets are maintained on a daily standardized base diet consisting of a mix of commercial products (Harlan Teklad marmoset purified diet, Purina Mazuri, and Zupreem marmoset diet; Table 1). Marmosets in the colony receive limited dietary enrichment items including cheerios, marshmallows and peanuts. Eligibility criteria for the study included animals that were not receiving intervention including probiotics, antibiotics, or rapamycin treatment, and had no recent medical concerns. At the time of the study the Barshop colony consisted of 5 males and 3 females over the age of 10, and 7 males aged 8–9 that met the eligibility criteria. The colony also had 23 males and 23 females that were considered young adult age (2–5) that met eligibility criteria. Given the limitation in number of geriatric female marmosets in our population, this analysis focused on determining whether geriatric male marmoset microbiomes differed from young marmosets. While there is no evidence in the human literature that male and female gut microbiota populations differ significantly, potential sex differences have not been explored in geriatric populations and it remains unclear what effects the changes in sexually specific endocrine cycles might have on the microbiome (Duncan, Colman, & Kramer, 2012; Jašarević, Morrison, & Bale, 2016). In this analysis, we assessed 10 geriatric males (8+ years; mean (± SD) age was 10.2 (±2.03) years) and 10 young adult males (2–5 years; mean 3.1 (±0.7) years) which were randomly

selected from the available animals to assess intestinal microbial diversity. None of these animals had a history of disease, diarrhea or dietary shift; all known medical history is outlined in Table 2. A fresh stool sample was collected from each animal, placed in a sterile collection tube and frozen at −80˚ C until shipment for analysis.

Laboratory Procedures

Stool sample sequencing and analysis were completed by Second Genome, Inc., San Francisco, CA. Nucleic acid isolation was performed with the MoBio PowerMag® Microbiome kit (Carlsbad, CA) optimized on the KingFisher Nucleic Acid Purification System (Thermo Fisher, Waltham, MA) for high-throughput processing (Qiagen. PowerMag Microbiome RNA/DNA Isolation Kit). All samples were quantified via the Qubit® Quant-iT dsDNA High Sensitivity Kit (Invitrogen, Life Technologies, Grand Island, NY). To enrich the sample for bacterial 16S V4 rDNA region, DNA was amplified with 515F-806R primers that were tailed with sequences to incorporate Illumina® (San Diego, CA) adapters and indexing barcodes(Caporaso et al., 2011). Each PCR product was quantified using a fluorometric method (Qubit from Invitrogen, Life Technologies, Grand Island, NY), and equimolar amounts were pooled for sequencing. The pooled library was loaded onto the Illumina MiSeq® platform and the amplicons were sequenced using a 250 bp paired-end protocol.

Data and Statistical Analysis

An open reference operational taxonomic unit (OTU) picking strategy was used, such that all sequences could be assigned to a strain OTU. First, sequenced paired-end reads were merged and resulting sequences were compared to a reference database at Second Genome (StrainSelect) using USEARCH (Edgar, 2010). All sequences matching to a unique strain with an identity ≥99% were assigned a strain OTU, which represents ≥99% identical match to a biological sequence within the database. A difference of ≥0.25% between the identity of the best hit and the second best hit was required (e.g., 99.75 versus 99.5). For each strain OTU, one of the matching reads was selected as representative and all sequences were mapped by USEARCH against the strain OTU representatives to calculate strain abundances. The remaining non-strain sequences were quality filtered and de-replicated with USEARCH. Sequences that did not match the reference database then underwent de novo OTU clustering using the UPARSE clustering algorithm, which clusters sequences at 97% similarity (Edgar, 2013). The 97% threshold was derived from a study that found that most strains had 97% 16S rRNA sequence similarity (Konstantinidis & Tiedje, 2005). The UPARSE clustering algorithm comprises a chimera filtering and discards likely chimeric OTUs. All non-strain sequences that passed the quality filtering were mapped to the representative consensus sequences to generate an abundance table for de novo OTUs. All generated OTU sequences were assigned taxonomic classification via Mothur’s bayesian classifier,(Schloss et al., 2009) trained against the Greengenes reference database of 16S rRNA gene sequences clustered at 99% (McDonald et al., 2012). Removal of spurious OTUs was completed by independent filtering, such that OTUs that were seen in at least 10% of the dataset were kept.

Statistical analyses were conducted using R® statistical language and environment. Sample richness was estimated based on the number of OTUs present in a sample. Shannon diversity calculations accounted for OTU richness and relative abundance (Shannon, 1948). The Wilcoxon signed rank test was used to compare alpha diversity between groups. Abundance-weighted sample pair-wise differences were calculated using the Bray-Curtis dissimilarity (Bray & Curtis, 1957). PERMANOVA was used to determine if age significantly contributed to beta diversity of samples. Univariate differential abundance of OTUs was tested using a negative binomial noise model for the overdispersion and Poisson process intrinsic to these data, as implemented in the DESeq2 package (Love, Huber, & Anders, 2014), and described for microbiome applications (McMurdie & Holmes, 2014). DESeq was run under default settings and q-values calculated with the Benjamini-Hochberg procedure to correct p-values, controlling for a false discovery rate of 0.25.

A hierarchical clustering of the samples was used to graphically summarize the relationship between geriatric and young adult marmoset samples. Samples from the distance matrix were clustered hierarchically using the Ward2 method.

Results

Sequencing Summary

Prior to filtering, the number of OTUs and sequences generated were 641 and 3,267,331, respectively. A total of 467 OTUs from 3,257,446 sequences passed sample quality check and were used for downstream analyses; 99.84% of all sequences were able to be classified at the phylum, class, and order levels. Only 75.76% and 21.74% of OTUs were classified at the genus and species levels, respectively. Sample library sizes ranged from 137,270 to 198,201 reads in geriatric marmoset samples and 149,348 to 176,782 in the young adult marmoset samples.

Sample Diversity by Age Group

Figure 1 displays alpha diversity measures. Mean (± standard deviation) OTU richness was not statistically different between geriatric (287 ± 24) and young adult (272 ± 37) samples (p=0.4490). However, Shannon diversity was significantly lower in geriatric (3.15 ± 0.37) compared to young adult (3.46 ± 0.33) samples (p=0.0191). Hierarchical clustering largely separated samples by age (Figure 2).

Sample Composition by Age Group

Age was associated with significant changes in relative abundance of certain bacterial phyla and families. Table 3 provides the proportional abundance of the 5 most common bacterial phyla and 8 families identified. At the phylum level, geriatric marmosets had a significantly higher mean abundance of Proteobacteria (0.22 vs. 0.09; p=0.0233) and lower abundance of Firmicutes (0.15 vs. 0.19; p=0.0032) compared to young adult marmosets. At the family level, geriatric marmosets had a significantly higher abundance of Succinivibrionaceae (0.16 vs. 0.01; p=0.0191) and lower abundance of Porphyromonadaceae (0.07 vs. 0.11; p=0.0494). Age significantly contributed to beta diversity of the samples (PERMANOVA p- =0.0190).

Discussion

Variability in microbiome diversity has been associated with differences in nonhuman primate environmental exposure, and dietary preferences, as well as human aging, obesity, and pathogenic disease state. Investigating causality and associations of microbiome change within the human population, or in wild primate populations, is broadly confounded by the inability to control for these environmental and dietary exposures. Further, it is difficult to determine the associations of microbial dysbiosis with human disease due to the inability to control for, or often account for pharmaceutical treatments. In order to determine whether changes in microbial diversity are associated with phenotypic aging, it is necessary to examine microbiome diversity in an animal model, preferably a closely related nonhuman primate species, which can be evaluated in captivity in a somewhat controlled exposure environment. This cross-sectional analysis of young adult and geriatric male marmosets identified significant differences in gut microbiome diversity and composition, with partial clustering of samples by age. Specifically, we found a less diverse microbiota that favored expansion of Proteobacteria and a reduction in the dominant Firmicutes phylum in geriatric marmosets compared to younger adult marmosets. To our knowledge, this is the first study to evaluate the effect of old age on the microbiome in a nonhuman primate.

Microbiome changes noted in this study mimic trends commonly seen in human studies. In healthy human adults, the gut microbiota measured at the bacterial phyla level is relatively stable, though bacterial species can greatly differ between individuals (Yatsunenko et al., 2012). Despite this variation, the overall functional capacity of the microbiome is similar across healthy persons (Qin et al., 2010). In contrast, the microbiome becomes less diverse in the elderly, with microbiota composition significantly correlated with measures of frailty, comorbidities, and inflammation (Claesson et al., 2012). Increasing populations of pathobionts have been found in the gut microbiota of centenarians with increased prevalence of Bacteriodetes and Proteobacteria in comparison to young adults (Rampelli et al., 2013) (Biagi et al., 2010).

The etiology of age-related microbiome changes is not well-defined; however, associations seen between dysbiosis and inflammatory conditions suggest a possible explanation. First, commensal bacteria are a critical regulator of the inflammatory response to acute injury and infection (Belkaid & Hand, 2014). Numerous animal studies and some human studies have noted increased inflammatory markers, as well as inflammatory conditions due to dysbiosis (Buford, 2017). Inflammatory conditions, like inflammatory bowel diseases, are associated with a reduction in species diversity and change in the abundance of bacterial taxa; obligate anaerobes from Bacteroidetes and Firmicutes decrease in abundance while facultative anaerobes from the Proteobacteria phyla are enriched (Buford, 2017). These changes could potentiate the inflammatory response. On the other hand, age-related changes, ranging from gut motility and cellular function to broad shifts in environmental exposure, could contribute to dysbiosis. Exposure to medications, like antibiotics, can also severely disrupt the microbiota and it may never fully recover to its original composition (Dethlefsen, Huse, Sogin, & Relman, 2008; Dethlefsen & Relman, 2011). Broad-spectrum antibiotic use has increased among older adults in recent years, which could contribute to alterations in the microbiome seen in this group (Lee GC, 2014). Physical activity and diet, including dietary

preference and nutrient availability, may also change with age, both of which can impact microbiome composition (Blaser & Falkow, 2009). These environmental confounds make it particularly difficult to determine the causal links between microbiome health and healthy aging in human aging populations. Evaluating microbial health in a nonhuman primate model in association with aging is the first step in determining whether evaluations of microbial health may serve as a biomarker for increased risk of unhealthy aging.

While it is impossible to control for all environmental factors in a study examining nonhuman primates, this study examined animals that had lived in the same stable environment for several years, had the same diet that did not change with age, and had known medical exposure over the course of their lifetime. The males in this study were housed singly or with female pair-mate, none of the lived with each other, and none were closely related to each other. The males in this study had no previous exposure to oral antibiotics throughout their lifetime. While the geriatric males had almost all undergone vasectomy several years prior to this study, antibiotics are not standardly given during this procedure. The males examined originated from different colonies but had been in the Barshop colony for at least one year prior to sampling. Only one male had a medical history that included exposure to probiotic treatment, which was provided to the entire family group that he was housed with at the time. Three young males had participated in a study that included dosing with acarbose for two weeks one year prior to this microbiome study. Given the medical histories for the animals that were sampled and the fact that we found a significant alteration in the microbiome associated with age it suggests that changes in microbial diversity in aging subjects may be independent of changes associated with environmental and lifestyle shifts. There are no known husbandry, housing or dietary components that can be used to explain why three geriatric animals clustered more closely with the younger animals. As a short-term cross sectional study that only examined male marmosets, it is possible that the differences found between these young adult and geriatric marmosets do not reflect longitudinal microbiome changes, and it might not be generalizable to female subjects, which will need to be evaluated in the future as geriatric female marmosets become available. However, these findings strongly suggest that further work is needed to evaluate microbiome diversity in geriatric populations of nonhuman primate models in order to determine the causes of declining microbial diversity in aging individuals and to determine how shifts in diversity relate to measures of healthspan. Of particular importance for marmoset aging will be the ability to evaluate the associations between changes in microbial diversity and gut functionality associated with digestibility. Microbiome shifts with age may be a precursor biomarker for age related health decline, and the ability to identify specific targets for changes in diversity may help us to identify individuals at risk for more rapid decline. In summary, geriatric marmosets had significantly altered microbiome diversity and composition compared to young marmosets. These findings indicate a potential role of the gut microbiome in aging nonhuman primate and human health. Further studies are needed to test microbiome-targeted therapies to improve healthspan and lifespan. This work broadens our knowledge of microbial diversity and its association with aging, aside from environmental confounds. This is particularly important for the development of biomarkers for healthy aging as well as potentially offering therapeutic targets for healthy aging interventions.

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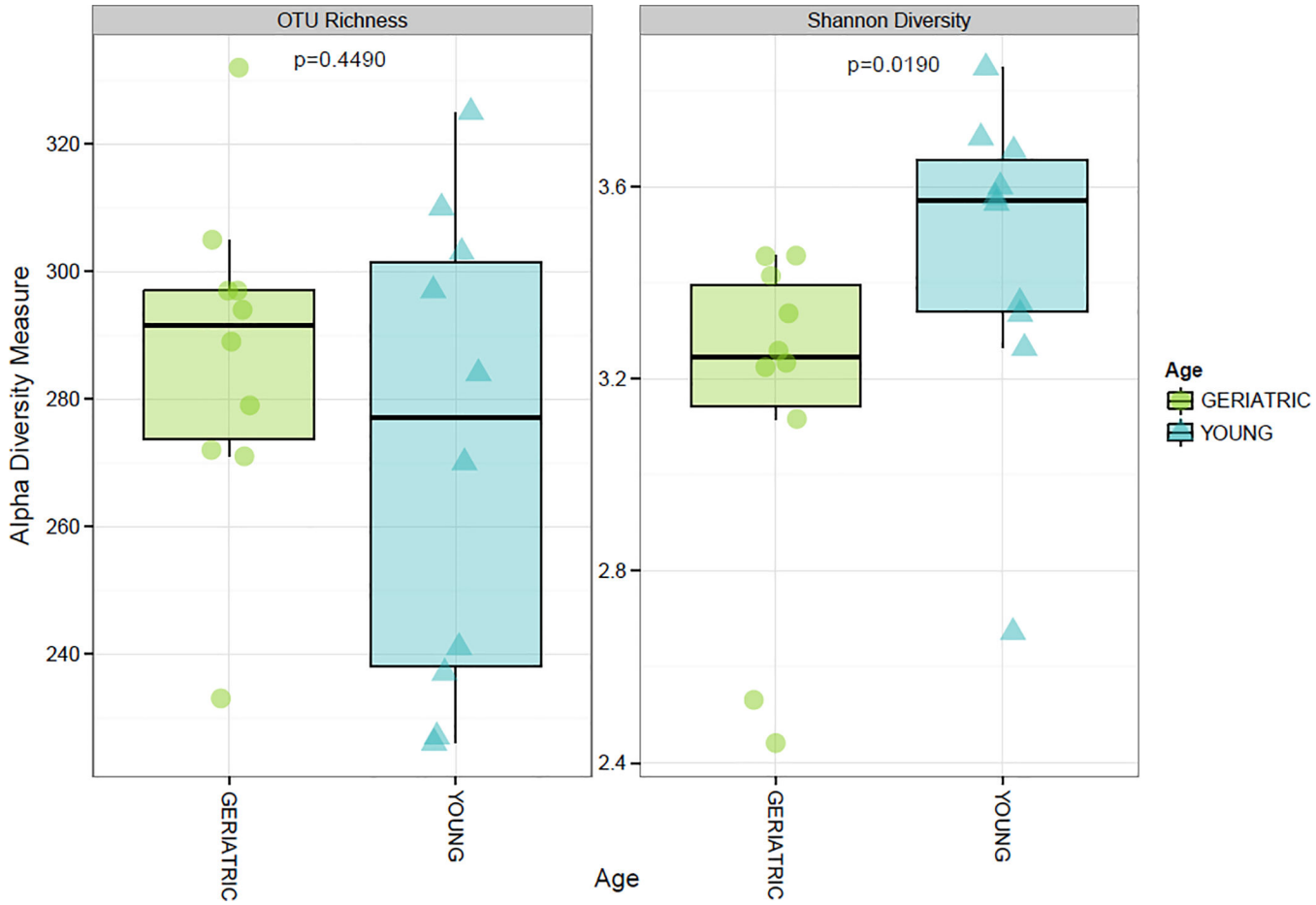

Figure 1.
Comparison of alpha diversity measures between geriatric and young adult marmoset fecal microbiome samples.

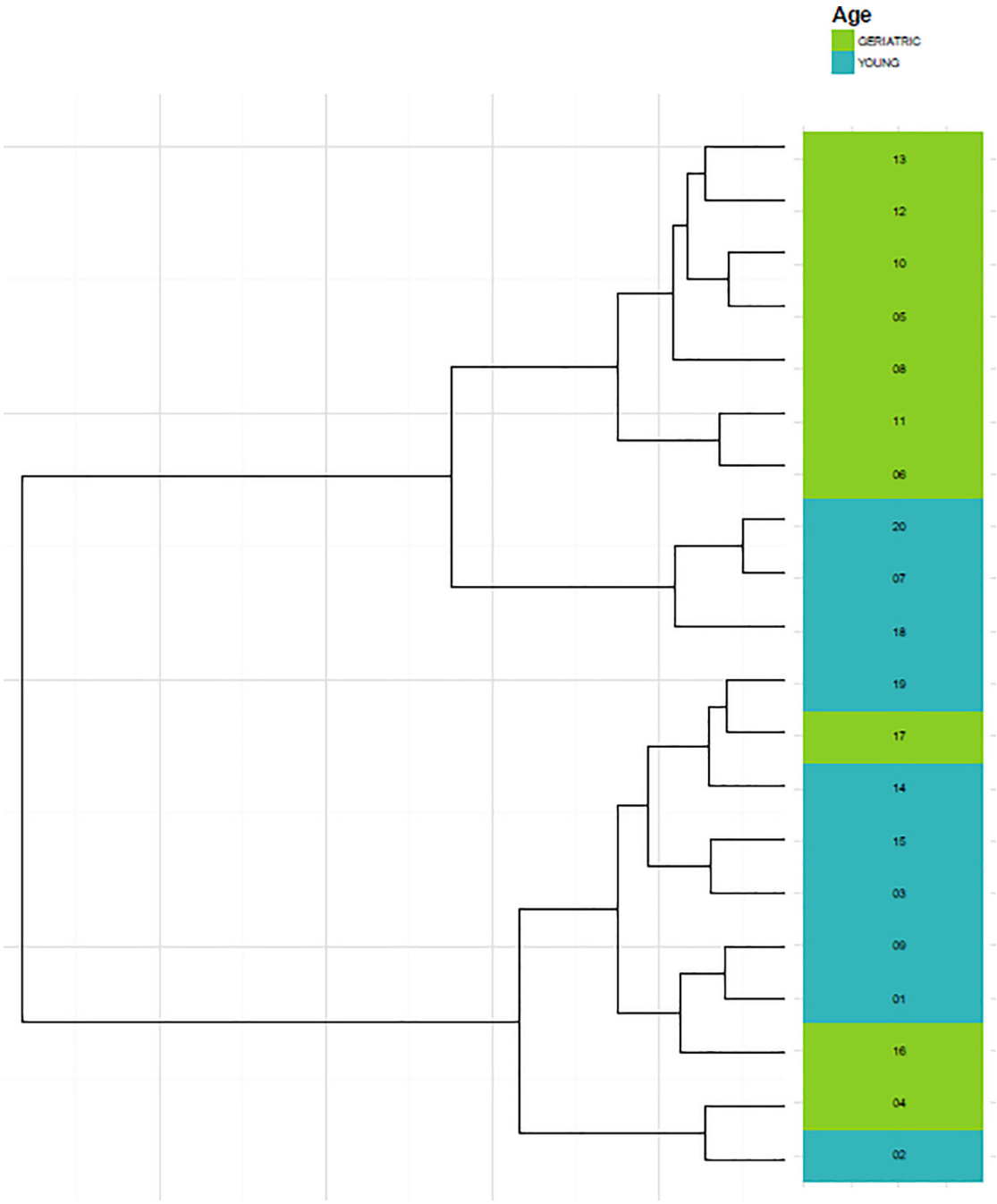

Figure 2.
Hierarchical clustering of geriatric and young adult marmoset microbiome samples

Table 1.

Macronutrient composition of marmoset base diets

Nutrient	Harlan Purified	Mazuri	Zupreem
Protein %	15.4	21.3	8.5
Fat %	13.8	7.8	2.5
Carbohydrate %	70.8	70.9	89
Energy (kcal/g)	3.6	3.4	3.4

Table 2.

Health history of the young adult and geriatric marmosets

Animal ID	Age	Housing	Medical History
14	2.0	paired	Barshop born
2	2.0	paired	SNPRC born, transferred to Barshop 2016
20	2.6	single	Barshop born
1	2.9	single	Barshop born, given acarbose two weeks 2016
19	3.1	paired	Barshop born
15	3.2	paired	Barshop born
9	3.3	single	Barshop born, given acarbose two weeks 2016
7	3.7	paired	Barshop born, given acarbose two weeks 2016
18	3.8	family	Barshop born, family given probios Feb 2014
3	4.1	paired	Barshop born
17	8.1	paired	Barshop born, vasectomy July 2016
4	8.2	single	Barshop born, vasectomy July 2016
8	8.6	paired	NEPRC born, transferred to SNPRC 2014, transferred to Barshop 2015, vasectomy Oct 2016
5	9.2	paired	SNPRC born, transferred 2014, vasectomy Oct 2016
13	9.2	paired	NEPRC born, transferred to SNPRC 2014, vasectomy 2014, transferred to Barshop 2015
10	9.7	paired	NEPRC born, transferred to SNPRC 2014, vasectomy 2014, transferred to Barshop 2015
12	10.5	paired	NEPRC born, transferred to SNPRC 2014, vasectomy 2014, transferred to Barshop 2015
6	11.9	paired	NEPRC born, transferred to SNPRC 2014, vasectomy 2014, transferred to Barshop 2015
11	12.1	paired	NEPRC born, transferred to SNPRC 2014, vasectomy 2014, transferred to Barshop 2015
16	14.3	paired	SNPRC born, transferred to Barshop 2013

Barshop = UT Health Science Center Barshop Institute Colony, SNPRC = Southwest National Primate Research Center, NEPRC = New England Primate Research Center

Table 3.

Comparison of select taxa relative abundance between geriatric and young adult marmoset samples

Bacteria	Geriatric (n=10)	Young Adult (n=10)	Raw p-value	Adjusted p-value[a]
Phylum, mean (SD)				
Bacteroidetes	0.35 (0.06)	0.349 (0.1)	0.7055	0.2222
Actinobacteria	0.21 (0.1)	0.254 (0.2)	0.5967	0.1667
Firmicutes	0.15 (0.04)	0.2 (0.03)	**0.0032**	**0.0278**
Proteobacteria	0.22 (0.11)	0.09 (0.05)	**0.0233**	**0.0556**
Fusobacteria	0.08 (0.1)	0.12 (0.15)	0.7055	0.2500
Family, mean (SD)				
Bifidobacteriaceae	0.12 (0.05)	0.15 (0.11)	0.9397	0.2500
Bacteroidaceae	0.07 (0.05)	0.12 (0.06)	0.0696	**0.1111**
Coriobacteriaceae	0.09 (0.05)	0.10 (0.05)	0.4497	0.1667
Fusobacteriaceae	0.08 (0.10)	0.11 (0.15)	0.7055	0.2222
Porphyromonadaceae	0.07 (0.04)	0.11 (0.06)	**0.0494**	**0.0556**
Prevotellaceae	0.13 (0.14)	0.04 (0.06)	0.1509	0.1389
Succinivibrionaceae	0.16 (0.11)	0.01 (0.01)	**0.0191**	**0.0278**
Veillonellaceae	0.07 (0.02)	0.08 (0.02)	0.5453	0.1944

[a]P-values adjusted using the Benjamini-Hochberg procedure and a false discovery rate of 0.25; significance level set at 0.0556 for phyla comparisons and 0.1111 for the family comparisons