APOE Genetics Influence Murine Gut Microbiome

Diana J. Zajac  
University of Kentucky College of Medicine

Stefan J. Green  
Rush University Medical Center

Lance A. Johnson  
University of Kentucky College of Medicine

Steven Estus (✉️ steve.estus@uky.edu)  
University of Kentucky College of Medicine  https://orcid.org/0000-0001-6900-6003

Research

Keywords: Alzheimer's, microbiome, APOE, sex differences

DOI: https://doi.org/10.21203/rs.3.rs-746611/v1

License: ☭ This work is licensed under a Creative Commons Attribution 4.0 International License.  
Read Full License
APOE genetics influence murine gut microbiome

Diana J. Zajac¹, Stefan J. Green²#, Lance A. Johnson¹, and Steven Estus¹∗

¹Department of Physiology and Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY
²Genome Research Core, Research Resources Center, University of Illinois at Chicago, Chicago, IL
# Current address: Genomics and Microbiome Core Facility, Rush University Medical Center, Chicago, IL

* Corresponding author: University of Kentucky, Department of Physiology, 789 S. Limestone, Rm. 537, Lexington, Kentucky 40536. Tel.: +1 859-218-3858; fax: +1 859-323-2866.

E-mail address: steve.estus@uky.edu (Steven Estus).

Email Addresses

• Diana Zajac: dianazajac228@uky.edu
• Stefan Green: greendna@uic.edu
• Lance Johnson: Johnson.lance@uky.edu
• Steven Estus: steve.estus@uky.edu

Keywords

Alzheimer’s, microbiome, APOE, sex differences
Abstract

Background: Apolipoprotein E (APOE) alleles impact pathogenesis and risk for multiple human diseases, making them primary targets for disease treatment and prevention. Previously, we and others reported an association between APOE alleles and the gut microbiome. Here, we tested whether these results are confirmed by using mice that were maintained under ideal conditions for microbiome analyses.

Methods: To model human APOE alleles, this study used APOE targeted replacement (TR) mice on a C57Bl/6 background. To minimize genetic drift, APOE3 mice were crossed to APOE2 or APOE4 mice prior to the study, and the resulting heterozygous progeny crossed further to generate the study mice. To maximize environmental homogeneity, mice with mixed genotypes were housed together and used bedding from the cages was mixed and added back as a portion of new bedding. Fecal samples were obtained from mice at three-, five- and seven-months of age, and microbiota analyzed by 16S ribosomal RNA gene amplicon sequencing. APOE2/E2 and APOE2/E3 mice were categorized as APOE2, APOE3/E4 and APOE4/E4 mice were categorized as APOE4, and APOE3/E3 mice were categorized as APOE3. Linear discriminant analysis of Effect Size (LefSe) identified taxa associated with APOE status, depicted as cladograms to show phylogenetic relatedness. The influence of APOE status was tested on alpha-diversity (Shannon H index) and beta-diversity (principal coordinate analyses and PERMANOVA). Individual taxa associated with APOE status were identified by classical univariate analysis. Whether findings in the APOE mice were replicated in humans was evaluated by using published microbiome genome wide association data.

Results: Cladograms revealed robust differences with APOE in male mice and limited differences in female mice. The richness and evenness (alpha-diversity) and microbial community composition (beta-diversity) of the fecal microbiome was robustly associated with
APOE status in male but not female mice. Classical univariate analysis revealed individual taxa that were significantly increased or decreased with APOE, illustrating a stepwise APOE2-APOE3-APOE4 pattern of association. The Clostridia class, Clostridiales order, Ruminococaceae family and related genera increased with APOE2 status. The Erysipelotrichia phylogenetic branch increased with APOE4 status, a finding that extended to humans.

Conclusions: In this study wherein mice were maintained in an ideal fashion for microbiome studies, gut microbiome profiles were strongly and significantly associated with APOE status in male APOE-TR mice. Erysipelotrichia in particular appears to increase with APOE4 in both mice and humans. Further evaluation of these findings in humans, as well as studies evaluating the impact of the APOE-associated microbiota on disease-relevant phenotypes, will be necessary to determine if alterations in the gut microbiome represents a novel mechanism whereby APOE alleles impact disease.
Introduction

Apolipoprotein E (APOE) alleles impact multiple facets of the human condition, ranging from Alzheimer’s Disease (AD) to cardiovascular disease, metabolic syndrome, obesity, fertility and longevity (reviewed in (1)). The three primary APOE alleles include APOE3, which has a 78% minor allele frequency, as well as APOE4 and APOE2, with minor allele frequencies of 14 and 8%, respectively. Regarding AD, APOE2 reduces AD risk while APOE4 strongly increases AD risk, both relative to APOE3 (reviewed in (2)). This association has prompted intense evaluation of possible mechanism(s) underlying APOE effects in AD, resulting in APOE allelic association with amyloid-beta (Aβ) clearance, Aβ aggregation and astrocyte stress (3-8). In the periphery, APOE2 is associated with decreased low-density lipoprotein (LDL) cholesterol, whereas APOE4 is associated with increased LDL cholesterol, relative to APOE3. While this may account for APOE association with cardiovascular disease, the mechanisms underlying APOE allelic effects on glucose metabolism, inflammation and innate immunity are unclear (2). Elucidating these differential actions of APOE alleles may provide insights to these processes.

Several studies have suggested a relationship between APOE status, the gut microbiome and AD neuropathology. First, APOE-deficient mice display microbiome differences relative to wild-type mice (9). Second, APOE4-targeted replacement (TR) mice were more resistant to gastrointestinal Cryptosporidium infection than APOE3 mice (10). Third, the APOE4 allele in humans was associated with better defense against childhood diarrheal diseases in lower income countries (11-13). Fourth, we and others have recently reported microbiome differences in a comparison of APOE3 and APOE4-TR mice (14-16). Lastly, several reports have found that Aβ-burden in murine models is reduced in gnotobiotic mice or mice treated with antibiotics (17-20). The mechanism(s) whereby APOE alleles influence the gut microbiome are unclear, although APOE4 has been associated with a greater inflammatory response to lipopolysaccharide (LPS), a microbiome product common to all gram-negative bacteria, in both humans and mice (21, 22).
Here, we sought to test the reproducibility of prior studies evaluating APOE genetics and the gut microbiome. Moreover, we incorporated improvements into study design that included backcrossing the APOE-TR mice to obviate possible genetic drift, maintaining mice with mixed genotypes in the same cages to minimize possible cage effects, and mixing used bedding between cages to ensure a homogenous microbial environment among cages.

**Materials and Methods**

**Mice:** APOE3-TR (23, 24) male mice were crossed to APOE4 and APOE2 female mice to produce APOE2/E3 and APOE3/E4 heterozygous offspring. These mice were then crossed to generate 76 experimental mice that included APOE2/E2 (N=6), APOE2/E3 (N=12), APOE3/E3 (N=5), APOE3/E4 (N=8), and APOE4/E4 (N=4) female mice and APOE2/E2 (N=5), APOE2/E3 (N=7), APOE3/E3 (N=13), APOE3/E4 (N=11), and APOE4/E4 (N=5) male mice. Genotypes were determined by TaqMan SNP assays (Thermo). At weaning, mice were separated by sex and housed as mixed genotypes, 2-5 mice per cage (average of 3.7 ± 1.4 (mean ± SD)). Mice were maintained on Teklad Global 18% Protein Rodent Diet. To minimize potential confounding effects of coprophagy (mice feeding partially on their feces) (25), approximately 20% of the new bedding was a mixture of used bedding from all the cages. Feces were obtained from this cohort of mice at three-, five- and seven-months of age. To obtain feces, mice were temporarily removed from their cage and placed into a clean Styrofoam cup. Fresh fecal pellets were stored at -80˚C until DNA isolation. All methods were approved by University of Kentucky Institutional Animal Care and Use Committee.

**Microbiome Analysis:** Fecal DNA was isolated by using a QIAamp PowerFecal Pro DNA Kit (QIAGEN). Genomic DNA was polymerase chain reaction (PCR) amplified with primers CS1_515F and CS2_806R (modified from the primer set employed by the Earth Microbiome Project (EMP; GTGYCAGCMGCCGCGGTAA and GGACTACNVGGGTWTCTAAT) targeting the V4 regions of microbial small subunit ribosomal RNA genes. Amplicons were generated using a
two-stage PCR amplification protocol as described previously (26). The primers contained 5’ common sequence tags (known as common sequence 1 and 2, CS1 and CS2). First stage PCR amplifications were performed in 10 microliter reactions in 96-well plates, using MyTaq HS 2X mastermix (Bioline). PCR conditions were 95°C for 5 minutes, followed by 28 cycles of 95°C for 30”, 55°C for 45” and 72°C for 60”.

Subsequently, a second PCR amplification was performed in 10 microliter reactions in 96-well plates. A mastermix for the entire plate was made using MyTaq HS 2X mastermix. Each well received a separate primer pair with a unique 10-base barcode, obtained from the Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA; Item# 100-4876). Cycling conditions were: 95°C for 5 minutes, followed by 8 cycles of 95°C for 30”, 60°C for 30” and 72°C for 30”. Samples were then pooled, purified, and sequenced on an Illumina MiniSeq platform employing paired-end 2x153 base reads. Fluidigm sequencing primers, targeting the CS1 and CS2 linker regions, were used to initiate sequencing. De-multiplexing of reads was performed on instrument. Library preparation, pooling, and sequencing were performed at the University of Illinois at Chicago Genome Research Core (GRC) within the Research Resources Center (RRC). Forward and reverse reads were merged using PEAR (27) and trimmed based on a quality threshold of $p = 0.01$. Ambiguous nucleotides and primer sequences were removed and sequences shorter than 225 bp were discarded. Chimeric sequences were identified and removed using the USEARCH algorithm with a comparison to the Silva 132_16S reference database (28, 29). Amplicon sequence variants (ASVs) were identified using DADA2 (30) and their taxonomic annotations determined using the UCLUST algorithm and Silva 132_16S reference with a minimum similarity threshold of 90% (28, 29).

This sequencing effort yielded 10,162,042 reads. Raw sequence data files were submitted in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI). Two samples with fewer than 30,000 reads each were discarded. Since APOE effects may be sex dependent (16), microbiomes from male and female mice were
analyzed separately. Average read counts per sample for the three-month males was 48,725, three-month females was 49,499, five-month males was 45,985, five-month females was 49,939, in seven-month males was 50,975, in seven-month females was 51,931. Using MicrobiomeAnalyst (31) (updated version February 2021), samples were rarified to the minimum library size, which for three-month males was 36,421, three-month females was 37,069, five-month males to 30,738, five-month females to 35,467, seven-month males to 35,452, and seven-month females to 36,730. Low abundance ASVs were removed, i.e., ASVs with < three counts in > 90% of the samples were removed, and low variance ASVs were also removed, i.e., ASVs whose inter-quantile range was in the lowest 10% (31). These corrections reduced the number of ASVs from 263 to 63 ASVs in three-month males and females, 67 in five-month females, 69 in five-month males, 68 in seven-month females and 66 in seven-month males. Count data was normalized with a centered log-ratio transformation. APOE2/E3 heterozygous mice were grouped with APOE2/E2 mice while APOE3/E4 mice were grouped with APOE4/E4 as described in other APOE studies (32, 33). This resulted in 12 APOE2, 13 APOE3 and 16 APOE4 male mice and 18 APOE2, five APOE3 and 12 APOE4 female mice.

Bacteria associated with APOE were identified by a linear discriminant analysis effect size (LefSe) approach (34). Significance thresholds were set to 0.05 for the alpha values for Kruskal-Wallis/Wilcoxon tests and 2.0 for the logarithmic linear discriminant analysis (LDA) score, using a one-against-all multi-class analysis approach. These results were then plotted as a cladogram to document the phylogenetic relatedness of APOE allelic associations with the bacteria at each taxonomic level.

Alpha-diversity was assessed using the Shannon H diversity index (35) with APOE statistical significance determined by Kruskal-Wallis tests. Beta-diversity was assessed using Principal Coordinates Analysis (PCoA) of Bray-Curtis matrices with statistical significance determined by Permutational Multivariate Analysis of Variance (PERMANOVA) (36). Taxonomic levels that associate with APOE status were determined using a classical univariate analysis with
a Kruskal–Wallis test. A false discovery rate (FDR) approach was used to correct for multiple
testing (31). Heatmaps of family-level bacterial relative abundances were generated for male and
female mice as a function of APOE status by using the Ward analysis of variance clustering
algorithm that used Pearson Correlation Coefficient distance measures.

Results

To investigate the hypothesis that APOE is associated with gut microbial community
structure, we began by visualizing the results with cladograms wherein the taxa were subjected
to LefSe analysis as a function of APOE alleles. This robust approach provides a statistical and
visual means to capture the hierarchical relationships associated with APOE status (34). We
found robust gut microbiome profile differences in male mice compared to female mice at three-
months of age (Figure 1) as well as at five- and seven-months of age (Supplemental Figure S1A-
B). Overall, these results indicated that a subset of bacteria were consistently associated with
APOE status. In the following figures, we depict data from the mice at three-months of age with
analyses for all ages included within the Supplemental files.

Microbiome alpha- (within sample) diversity was assessed by the Shannon H index, a
measure of taxon richness and evenness (reviewed in (37)). An association between alpha-
diversity and APOE was not detected in female mice (Figure 2A, results for all ages in Table 1).
In contrast, male mice showed a stepwise trend towards higher alpha-diversity with APOE2-
APOE3-APOE4 at the genus level, and this trend became more defined and statistically
significant at every higher level through phylum, with genus and order depicted in Figure 2B (all
ages in Table 1). Hence, APOE was associated with alpha-diversity in male but not female mice.

Beta-diversity is a measure of between sample microbial communities based on their
composition. Beta-diversity was visualized by using PCoA based on Bray-Curtis distance
matrices (38-41), and analyzed using a PERMANOVA. We found that APOE status was
significantly associated with microbiome beta-diversity in male but not female mice, as shown in
Figure 2 C-D. Table 2 provides beta-diversity p-values and $R^2$ values for male and female mice at all ages. Overall, these results suggest that the microbiome is robustly associated with APOE genetics in male mice compared to female mice.

Variation in bacterial relative abundance per sample was visualized using heatmaps, as seen in Figure 3A-B. While cladograms identify bacterial phylogenetic branches that correlate with high abundance in association with a specific APOE status, heatmaps provide a per sample depth of information for each APOE status. Inspection of the heatmaps suggests that the data are relatively complex although some bacterial patterns of association with APOE status are discernible, e.g., Ruminococcaceae and Erysipelotrichaceae in male mice (Figure 3B).

The combination of LefSe analysis and cladogram visualization has been optimized for microbiome analysis (34). Another approach often used to analyze microbiome studies is a taxon-by-taxon classical univariate analysis using a Kruskal-Wallis test for significance with an FDR correction for multiple testing. To highlight the taxa most robustly associated with APOE, we applied a classical univariate analysis to identify results that were significant with both approaches. Classical univariate analysis of the three-month old female mice found no ASVs, genera, families, orders, classes, or phyla that were significantly associated with APOE. However, this approach applied to three-month old male mice found 12 ASVs that were significant as well as 12 genera, six families, five orders, five classes, and one phylum (statistics for all taxa in each age group are listed in Supplementary Tables S1.1-S3.4). A graphical representation of the findings in three-month old male mice is depicted in Figure 4, with the box plots organized per the relevant phylogenetic tree. Several bacteria showed stepwise associations with APOE on multiple taxonomic levels and were overall increased with APOE2. For example, the Clostridia class, Clostridiales order and two major families within this phylogenetic branch, Ruminococcaceae and Lachnospiraceae showed a decrease in their relative abundance from APOE2 to APOE3 to APOE4 (Figure 4). The most abundant genera within the Ruminococcaceae family significantly associated with APOE were Ruminiclostridium,
Ruminiclostridium_5 and Ruminiclostridium_9, which in aggregate represent approximately half of the Ruminococcaceae family (Figure 4). At five- and seven-months of age, other genera within Ruminococcaceae were associated with APOE (Supplemental Tables S1.1-S3.4). Genera within the other major family, Lachnospiraceae, that increased with APOE2 were Acetifactor and Lachnoclostridium (Figure 4).

In contrast, other taxa were increased with APOE4, most notably bacteria within the phylogenetic branch defined by the Erysipelotrichia class, confirming the findings from the LefSe cladograms (Figure 1). Bacteria within the Erysipelotrichia branch that were significantly associated with APOE included the order Erysipelotrichiales, its family Erysipelotrichaceae and its genera Turicibacter and Dubosiella (Figure 5, data for all ages shown in Supplementary Tables S1.1-S3.4). Consistent within this branch, bacterial relative abundance was near zero in the APOE2 mice, moderate in APOE3 and highly enriched in APOE4 (Figure 5). Hence, both the LefSe and classical approaches identified members of the Clostridia class as enriched in APOE2 mice while members of the Erysipelotrichia class were enriched in APOE4 mice.

To discern whether the Clostridiales and Erysipelotrichiales phylogenetic branches associated with APOE in this murine APOE-TR model are also associated with APOE in humans, we turned to a recent genome wide association study (GWAS) that evaluated the relationship between the gut microbiome and human polymorphisms (42). This meta-analysis included data from as many as 18,340 individuals (42). The only genetic locus that reached genome wide statistical significance was rs182549, which is associated with lactose intolerance. Interestingly, this SNP is modestly associated with the risk of Alzheimer’s disease (p=0.003, N=445,779) (43), consistent with the possibility that the gut microbiome may influence AD risk.

Focusing on APOE, the alleles of APOE2, APOE3 and APOE4 are defined by two SNPs, rs7412 and rs429358. The minor allele of rs7412 defines APOE2 while the minor allele of rs429358 determines APOE4 status. The Clostridiales and Erysipelotrichiales phylogenetic branches were not significantly associated with rs7412 (APOE2) at any phylogenetic level. However, the class
Erysipelotrichia, the order Erysipelotrichales and the family *Erysipelotrichaceae* were nominally associated with rs429358 (Table 3). For each of these taxa, the minor *APOE4* allele was associated with an increase in the relative abundance of these bacteria, reproducing the findings observed in the murine *APOE*-TR model.

**Discussion**

The primary finding reported here is that murine gut microbiome profiles are significantly associated with *APOE* status in a study wherein the *APOE*-TR mice were maintained in an optimized fashion for microbiome analyses. The microbiome association with *APOE* was observed in alpha- and beta-diversity, encompasses multiple bacterial lineages and was predominately in male mice. Both LefSe and classical univariate analyses identified specific taxa that were associated with *APOE*. This association occurred in a stepwise fashion in the mice with the progression from *APOE2*-APOE3-*APOE4*. The stepwise association between indices of the gut microbiome and *APOE2*-APOE3-*APOE4* reported here are reminiscent of *APOE* allelic association with other phenotypes ranging from LDL-cholesterol to AD risk (1, 2). Additionally, at least one of these associations, an increase in the Erysipelotrichia phylogenetic branch with *APOE4*, is also observed in the human gut microbiome. Overall, these findings confirm and extend prior reports that *APOE* genetics are associated with the gut microbiome (14-16).

To identify the impact of *APOE* alleles on the microbiome, we used several approaches in this study. These approaches included alpha-diversity, beta-diversity, LefSe and classical univariate analyses. Alpha- and beta-diversity analyses aggregate multiple variables to provide an assessment of overall microbiome diversity and of microbiome profile similarity, respectively. In contrast, LefSe and classical univariate analyses provide an indication of differences in the relative abundance of specific taxa between experimental groups. In this discussion, we will highlight the primary significant findings from these various analyses.
*APOE4* was associated with increased alpha-diversity as assessed by the Shannon H index. A stepwise progression was observed with lowest alpha-diversity in *APOE2* moderate in *APOE3* and highest in *APOE4*. Alpha-diversity is a measure of the number of distinct taxa and the evenness of these numbers across taxa. High alpha-diversity in the gut microbiome has been associated with improved gut health and microbiome homeostasis (reviewed in (37)). The *APOE4* association with increased alpha-diversity observed here is consistent with prior observations that *APOE4* is associated with better response to diarrheal infections in a third-world environment (12, 13). Indeed, the enrichment of *APOE4* in people indigenous to Amazonian basin has been proposed to be a result of evolutionary selection in this environment with insufficient sanitation (44).

A primary finding of this study was that both the LefSe and classical univariate analyses found that taxa within the Clostridia class were increased with *APOE2* status, confirming results from our prior study (14) and that of Tran et al (15). This phylogenetic branch included the Clostridiales order, *Ruminococcaceae* family and several genera within this family. The Clostridiales order was increased in *APOE2* mice compared to *APOE3* and *APOE4* mice. This was most robust in the three-month males with similar findings at five- and seven-months. The two major bacterial families within this order, *Ruminococcaceae* and *Lachnospiraceae*, were also both increased with *APOE2*. The stepwise fashion of the decline in *Ruminococcaceae* relative abundance from *APOE2* to *APOE3* to *APOE4* confirms the stepwise pattern seen previously (14, 16) and extends it along the phylogenetic branch from the Clostridiales class to associated genera, such as *Ruminiclostridium*, *Ruminiclostridium_5*, *Ruminiclostridium_9*. Interestingly, Tran et al. also reported an increase in relative abundance of the Clostridiales order and *Ruminococcaceae* family in *APOE2/E3* humans compared to *APOE3/E4* and *APOE4/E4* humans (15). This suggests that this increase in Clostridiales and *Ruminococcaceae* with *APOE2* may extend to humans. Two additional genera in the Clostridial class, within the *Lachnospiraceae* family, i.e., *Acetifactor* and *Lachnoclostridium*, also increased with *APOE2* status in the current study.
However, this finding was not replicated by Tran et al., who reported that *Lachnospiraceae* increased in *APOE4* mice compared to *APOE3* mice (15).

Our study strengthens the associations between *APOE* status and the Clostridiales order, *Ruminococcaceae* family and related genera, and the *Acetifactor* and *Lachnoclostridium* genera by demonstrating a stepwise pattern with *APOE* allelic status across the entire phylogenetic branch from the Clostridia class down to related genera. *Ruminococcaceae* and *Lachnospiraceae* are bacterial families that highly express genes responsible for the metabolism of resistant starches in the large intestine, generating short chain fatty acids (SCFA)s. The presence of SCFAs in the gut affect human health in general (reviewed in (45, 46)) and have been reported to promote microglial maturation and function in particular (47). Treatment with SCFAs has been shown to reduce microglial pro-inflammatory signals and promote a homeostatic profile that is neuroprotective (48-51). Considering these findings relative to disease pathology associated with the stepwise *APOE2-APOE3-APOE4* phenotype, we propose a tentative model wherein (i) *APOE2* is associated with an increase in the relative abundance of microbiome bacteria *Ruminococcaceae*, *Acetifactor* and *Lachnoclostridium*, relative to *APOE3* and *APOE4*, (ii) this shift in bacterial profile increases the production of SCFAs and (iii) this increase in SCFAs promotes microglial homeostasis and disease-ameliorating signaling, as suggested by robust genetic evidence (52-60), (reviewed in (61, 62)). While speculative, this model serves as a framework for future studies.

Another primary finding detected by both the LefSe and classical univariate analyses was that the *Erysipelotrichia* phylogenetic branch was significantly associated with *APOE* status in a stepwise *APOE2-APOE3-APOE4* pattern. This finding appeared to extend to humans and replicates the increase of the *Erysipelotrichaceae* family in *APOE4* mice that we observed previously (14). This parallels the association of the Erysipelotrichia class, Erysipelotrichales order and *Erysipelotrichaceae* family with the *APOE4* minor allele rs429358 in human GWAS data. Our current study also extends this finding from the Erysipelotrichia phylogenetic order to
its major genera, i.e. Turicibacter and Dubosiella. However, Tran et al. reported

*Erysipelotrichaceae* were significantly increased in APOE3 compared to APOE4 mice.

*Erysipelotrichaceae* has been shown to increase in animals fed a high-fat diet and to decrease in patients on a low-fat diet (63, 64). Hence, diet variation between the mice in our study and those of Tran et al. may account for the *Erysipelotrichaceae* difference, noting that our Teklad Global 18% (2018) chow has a fat content that accounts for 18% of total calories, whereas the RPM3, Special Diet Services chow used in the Tran et al study has a fat content that accounts for 12% of total calories (15). Since APOE genetics have been associated with BMI and obesity (1), there may be a complex interplay between diet, APOE genotype and relative abundance of *Erysipelotrichaceae* in the gut.

**Conclusions**

In this study in which mice were maintained with optimized conditions for microbiome analysis, we report a significant association between APOE status and gut microbiome profiles in three-month male mice that reproduces at five and seven months of age. The Clostridia class, Clostridiales order, its related family *Ruminococcaceae*, as well as related genera *Ruminoclostridium*, and *Acetifactor* and *Lachnoclostridium* of the *Lachnopsiraceae* family increase with APOE2, which may reflect an increase in resistant starch metabolism with APOE2, and a possible impact on SCFA levels. The Erysipelotrichia class, Erysipelotrichiales order, *Erysipelotrichaceae* family, and *Turicibacter* and *Dubosiella* genera increase with APOE4. The findings with the Erysipelotrichia phylogenetic branch appear to extend to humans. Understanding the effects of APOE genetics on the gut microbiome may provide novel approaches to counter deleterious APOE genetic effects on human disease.

**Ethics approval**
Animal studies were performed in compliance with the Institutional Animal Care and Use Committee at University of Kentucky.

**Competing interests**

The authors have no competing interests.

**Acknowledgements**

The authors appreciate helpful discussion by Drs. Jamie Sturgill and Sarah D’Orazio.

**Funding**

The authors acknowledge funding support from the NIH (R56-AG057589 and T32-GM118292).

**Authors’ Contributions**

DJZ purified DNA, analyzed sequencing results, generated figures and contributed to writing the manuscript. SJG performed PCR amplification, sequencing and sequencing analysis, LAJ optimized the microbiome study design, and SE contributed to data analysis and writing the manuscript.

**Consent for publication**

Each of the authors have reviewed the manuscript and approved it for publication.

**Data Availability**

Metadata, raw sequence data and taxa read counts are in the process of being submitted to BioProject. This will be completed before publication.

| Taxonomic level | 3 month p-values | 5 month p-values | 7 month p-values |
|-----------------|------------------|------------------|------------------|
|                 | Males            | Females          | Males            | Females          | Males            | Females          |
| genus           | 4.67E-01         | 5.78E-01         | 4.57E-01         | 7.86E-01         | 5.36E-01         | 7.71E-01         |
| family          | 5.78E-04         | 3.17E-01         | 1.95E-02         | 4.06E-01         | 5.01E-01         | 7.44E-01         |
| order           | 7.68E-05         | 7.68E-01         | 8.44E-02         | 6.08E-01         | 1.42E-01         | 2.81E-01         |
| class           | 7.68E-05         | 7.31E-01         | 7.25E-02         | 6.30E-01         | 1.35E-01         | 2.38E-01         |
| phylum          | 1.14E-05         | 6.49E-02         | 2.79E-01         | 9.53E-02         | 9.22E-01         | 2.46E-01         |
Table 1. Microbiome alpha-diversity was significantly associated with APOE status in three-month male but not female mice. P-values reflect nominal p values and were determined using Kruskal-Wallis tests.

| Taxonomic level | 3 month | 5 month | 7 month |
|-----------------|---------|---------|---------|
| Genus           |         |         |         |
| p-value         | <0.001  | <0.048  | <0.028  | <0.005  | <0.038 |
| R²              | 0.146   | 0.058   | 0.0085  | 0.123   | 0.095  |
| Family          |         |         |         |
| p-value         | <0.005  | <0.0496 | <0.080  | <0.060  | <0.001 | <0.201 |
| R²              | 0.165   | 0.052   | 0.088   | 0.164   | 0.077  |
| Order           |         |         |         |
| p-value         | <0.003  | <0.0688 | <0.089  | <0.786  | <0.006 | <0.165 |
| R²              | 0.206   | 0.036   | 0.100   | 0.179   | 0.091  |
| Class           |         |         |         |
| p-value         | <0.002  | <0.056  | <0.082  | <0.830  | <0.006 | <0.133 |
| R²              | 0.208   | 0.037   | 0.102   | 0.18    | 0.097  |
| Phylum          |         |         |         |
| p-value         | <0.028  | <0.023  | <0.046  | <0.055  | 0.102  |
| R²              | 0.122   | 0.069   | 0.023   | 0.055   | 0.102  |

Table 2. Microbiome beta-diversity significantly associated with APOE status in male, but not female, mice. The $R^2$ values represent the proportion of the variance captured by APOE alleles. The PERMANOVA results were derived from 999 permutations.

| Bacteria          | SNP     | Reference Allele | Effect Allele | beta | SE   | P Value | N   |
|-------------------|---------|------------------|---------------|------|------|---------|-----|
| Class: Erysipelotrichia | rs429358 | T                | C             | 0.032| 0.015| 0.035   | 18097|
| Order: Erysipelotrichales | rs429358 | T                | C             | 0.032| 0.015| 0.035   | 18097|
| Family: Erysipelotrichaceae | rs429358 | T                | C             | 0.032| 0.015| 0.035   | 18097|
| Genus: Turicibacter    | rs429358 | T                | C             | 0.001| 0.021| 0.87    | 8921|

Table 3. Bacteria in the Erysipelotrichia phylogenetic branch are nominally associated with rs429358. The positive beta values reflect that the bacterial taxa are increased with the
minor APOE4 allele of rs429358. These results are a combination of data from both men and women and are supplemental data from a large microbiome genetics study (42).

References

1. Martinez-Martinez AB, Torres-Perez E, Devanney N, Del Moral R, Johnson LA, Arbones-Mainar JM. Beyond the CNS: The many peripheral roles of APOE. Neurobiology of disease. 2020;138:104809.
2. Liu CC, Kanekiyo T, Xu H, Bu G. Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. Nature reviews Neurology. 2013;9(2):106-18.
3. Rodriguez GA, Tai LM, LaDu MJ, Rebeck GW. Human APOE4 increases microglia reactivity at Abeta plaques in a mouse model of Abeta deposition. Journal of neuroinflammation. 2014;11:111.
4. Conejero-Goldberg C, Gomar JJ, Bobes-Bascaran T, Hyde TM, Kleinman JE, Herman MM, et al. APOE2 enhances neuroprotection against Alzheimer’s disease through multiple molecular mechanisms. Molecular psychiatry. 2014;19(11):1243-50.
5. Tai LM, Mehra S, Shete V, Estus S, Rebeck GW, Bu G, et al. Soluble apoE/Abeta complex: mechanism and therapeutic target for APOE4-induced AD risk. Molecular neurodegeneration. 2014;9(1):2.
6. Ophir G, Meilin S, Efrati M, Chapman J, Karussis D, Roses A, et al. Human apoE3 but not apoE4 rescues impaired astrocyte activation in apoE null mice. Neurobiology of disease. 2003;12(1):56-64.
7. Zhu Y, Nwabuisi-Heath E, Dumanis SB, Tai LM, Yu C, Rebeck GW, et al. APOE genotype alters glial activation and loss of synaptic markers in mice. Glia. 2012;60(4):559-69.
8. Kim J, Jiang H, Park S, Eltorai AE, Stewart FR, Yoon H, et al. Haploinsufficiency of human APOE reduces amyloid deposition in a mouse model of amyloid-beta amyloidosis. The Journal of neuroscience. 2011;31(49):18007-12.
9. Saita D, Ferrarese R, Foglieni C, Esposito A, Canu T, Perani L, et al. Adaptive immunity against gut microbiota enhances apoE-mediated immune regulation and reduces atherosclerosis and western-diet-related inflammation. Scientific reports. 2016;6:29353.
10. Azevedo OG, Bolick DT, Roche JK, Pinkerton RF, Lima AA, Vitek MP, et al. Apolipoprotein E plays a key role against cryptosporidial infection in transgenic undernourished mice. PloS one. 2014;9(2):e89562.
11. Oria RB, Patrick PD, Blackman JA, Lima AA, Guerrant RL. Role of apolipoprotein E4 in protecting children against early childhood diarrhea outcomes and implications for later development. Medical hypotheses. 2007;68(5):1099-107.
12. Oria RB, Patrick PD, Oria MO, Lorntz B, Thompson MR, Azevedo OG, et al. ApoE polymorphisms and diarrheal outcomes in Brazilian shanty town children. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica [et al]. 2010;43(3):249-56.
13. Oria RB, Patrick PD, Zhang H, Lorntz B, de Castro Costa CM, Brito GA, et al. APOE4 protects the cognitive development in children with heavy diarrhea burdens in Northeast Brazil. Pediatric research. 2005;57(2):310-6.
14. Parikh IJ, Estus JL, Zajac DJ, Malik M, Maldonado Weng J, Tai LM, et al. Murine Gut Microbiome Association With APOE Alleles. Frontiers in immunology. 2020;11:200.
15. Tran TTT, Corsini S, Kellingray L, Hegarty C, Le Gall G, Narbad A, et al. APOE genotype influences the gut microbiome structure and function in humans and mice: relevance for Alzheimer’s disease pathophysiology. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2019;33(7):8221-31.
16. Maldonado Weng J, Parikh I, Naqib A, York J, Green SJ, Estus S, et al. Synergistic effects of APOE and sex on the gut microbiome of young EFAD transgenic mice. Molecular neurodegeneration. 2019;14(1):47.
17. Minter MR, Zhang C, Leone V, Ringus DL, Zhang X, Oyler-Castrillo P, et al. Antibiotic-induced perturbations in gut microbial diversity influences neuro-inflammation and amyloidosis in a murine model of Alzheimer's disease. Scientific reports. 2016;6:30028.
18. Harach T, Marungruang N, Duthilleul N, Cheatham V, Mc Coy KD, Frisoni G, et al. Reduction of Abeta amyloid pathology in APPPS1 transgenic mice in the absence of gut microbiota. Scientific reports. 2017;7:41802.
19. Bonfili L, Cecarini V, Berardi S, Scarpona S, Suchodolski JS, Nasuti C, et al. Microbiota modulation counteracts Alzheimer’s disease progression influencing neuronal proteolysis and gut hormones plasma levels. Scientific reports. 2017;7(1):2426.
20. Minter MR, Hinterleitner R, Meisel M, Zhang C, Leone V, Zhang X, et al. Antibiotic-induced perturbations in microbial diversity during post-natal development alters amyloid pathology in an aged APPSWE/PS1DeltaE9 murine model of Alzheimer's disease. Scientific reports. 2017;7(1):10411.
21. Vitek MP, Brown CM, Colton CA. APOE genotype-specific differences in the innate immune response. Neurobiology of aging. 2009;30(9):1350-60.
22. Gale SC, Gao L, Mikacenic C, Coyle SM, Rafaels N, Murray Dudenkov T, et al. APOe4 is associated with enhanced in vivo innate immune responses in human subjects. The Journal of allergy and clinical immunology. 2014;134(1):127-34.
23. Sullivan PM, Mezdour H, Aratani Y, Knouff C, Najib J, Reddick RL, et al. Targeted replacement of the mouse apolipoprotein E gene with the common human APOE3 allele enhances diet-induced hypercholesterolemia and atherosclerosis. The Journal of biological chemistry. 1997;272(29):17972-80.
24. Strattan LE, Britsch DRS, Calulot CM, Maggard RSJ, Abner EL, Johnson LA, et al. Novel Influences of Sex and APOE Genotype on Spinal Plasticity and Recovery of Function after Spinal Cord Injury. eNeuro. 2021;8(2).
25. Bo TB, Zhang XY, Kohl KD, Wen J, Tian SJ, Wang DH. Coprophagy prevention alters microbiome, metabolism, neurochemistry, and cognitive behavior in a small mammal. The ISME journal. 2020;14(10):2625-45.
26. Naqib A, Poggi S, Wang W, Hyde M, Kunstman K, Green SJ. Making and sequencing heavily multiplexed, high-throughput 16S ribosomal RNA gene amplicon libraries using a flexible, two-stage PCR protocol. Gene Expression Analysis. New York, NY: Humana Press; 2018. p. pp. 149-69.
27. Zhang J, Kobert K, Flouri T, Stamatakis A. PEAR: a fast and accurate Illumina Paired-End read merger. Bioinformatics. 2014;30(5):614-20.
28. Silva GG, Green KT, Dutilh BE, Edwards RA. SUPER-FOCUS: a tool for agile functional analysis of shotgun metagenomic data. Bioinformatics. 2016;32(3):354-61.
29. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26(19):2460-1.
30. Glockner FO, Yilmaz P, Quast C, Gerken J, Beccati A, Ciuprina A, et al. 25 years of serving the community with ribosomal RNA gene reference databases and tools. J Biotechnol. 2017;261:169-76.
31. Dhariwal A, Chong J, Habib S, King IL, Agellon LB, Xia J. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic acids research. 2017;45(W1):W180-W8.
32. Zhu H, Tucker HM, Grear KE, Simpson JF, Manning AK, Cupples LA, et al. A common polymorphism decreases low-density lipoprotein receptor exon 12 splicing efficiency and associates with increased cholesterol. Human molecular genetics. 2007;16(14):1765-72.
33. Tao Q, Ang TFA, DeCarli C, Auerbach SH, Devine S, Stein TD, et al. Association of Chronic Low-grade Inflammation With Risk of Alzheimer Disease in ApoE4 Carriers. JAMA Netw Open. 2018;1(6):e183597.
34. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome biology. 2011;12(6):R60.
35. Hammer Ø, Harper DAT, Ryan PD. Paleontological statistics software package for education and data analysis. Palaeontologia Electronica. 2001;4(1):9.
36. O, FG B, R K, P L, RB OH. vegan: Community Ecology Package. R package version. 2011;1:17-0.
37. Rinninella E, Raoul P, Cintoni M, Franceschi F, Miggiano GAD, Gasbarrini A, et al. What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. Microorganisms. 2019;7(1).
38. McMurdie PJ, Holmes S. Waste not, want not: why rarefying microbiome data is inadmissible. PLoS computational biology. 2014;10(4):e1003531.
39. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome biology. 2014;15(12):550.
40. McCarthy DJ, Chen Y, Smyth GK. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. Nucleic acids research. 2012;40(10):4288-97.
41. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS one. 2013;8(4):e61217.
42. Kuriyashikov A, Medina-Gomez C, Bacigalupe R, Radjabzadeh D, Wang J, Demirkan A, et al. Large-scale association analyses identify host factors influencing human gut microbiome composition. Nature genetics. 2021;53(2):156-65.
43. Jansen IE, Savage JE, Watanabe K, Bryois J, Williams DM, Steinberg S, et al. Genome-wide meta-analysis identifies new loci and functional pathways influencing Alzheimer’s disease risk. Nature genetics. 2019.
44. Vasunilashorn S, Finch CE, Crimmins EM, Vikman SA, Stieglitz J, Gurven M, et al. Inflammatory gene variants in the Tsimane, an indigenous Bolivian population with a high infectious load. Biodemography Soc Biol. 2011;57(1):33-52.
45. Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, Macia L. The role of short-chain fatty acids in health and disease. Advances in immunology. 2014;121:91-119.
46. Dalile B, Van Oudenhove L, Vervliet B, Verbeke K. The role of short-chain fatty acids in microbiota-gut-brain communication. Nature reviews Gastroenterology & hepatology. 2019;16(8):461-78.
47. Erny D, Hrabe de Angelis AL, Jaitin D, Wieghofer P, Staszewski O, David E, et al. Host microbiota constantly control maturation and function of microglia in the CNS. Nature neuroscience. 2015;18(7):965-77.
48. Wang P, Zhang Y, Gong Y, Yang R, Chen Z, Hu W, et al. Sodium butyrate triggers a functional elongation of microglial process via Akt-small RhoGTPase activation and HDACs inhibition. Neurobiology of disease. 2018;111:12-25.
49. Patnala R, Arumugam TV, Gupta N, Dheen ST. HDAC Inhibitor Sodium Butyrate-Mediated Epigenetic Regulation Enhances Neuroprotective Function of Microglia During Ischemic Stroke. Molecular neurobiology. 2017;54(8):6391-411.
50. Yamawaki Y, Yoshioka N, Nozaki K, Ito H, Oda K, Harada K, et al. Sodium butyrate abolishes lipopolysaccharide-induced depression-like behaviors and hippocampal microglial activation in mice. Brain research. 2018;1680:13-38.
51. Soliman ML, Puig KL, Combs CK, Rosenberger TA. Acetate reduces microglia inflammatory signaling in vitro. Journal of neurochemistry. 2012;123(4):555-67.
52. Wang Y, Cella M, Mallinson K, Ulrich JD, Young KL, Robinette ML, et al. TREM2 lipid sensing sustains the microglial response in an Alzheimer's disease model. Cell. 2015;160(6):1061-71.
53. Jonsson T, Stefansson H, Steinberg S, Jonsdottir I, Jonsson PV, Snaedal J, et al. Variant of TREM2 associated with the risk of Alzheimer's disease. The New England journal of medicine. 2013;368(2):107-16.
54. Jonsson T, Stefansson H, Ph DS, Jonsdottir I, Jonsson PV, Snaedal J, et al. Variant of TREM2 Associated with the Risk of Alzheimer's Disease. The New England journal of medicine. 2012.
55. Guerreiro R, Wojtas A, Bras J, Carrasquillo M, Rogaeva E, Majounie E, et al. TREM2 Variants in Alzheimer's Disease. The New England journal of medicine. 2012.
56. Sutherland MK, Yu C, Lewis TS, Miyamoto JB, Morris-Tilden CA, Jonas M, et al. Anti-leukemic activity of lintuzumab (SGN-33) in preclinical models of acute myeloid leukemia. mAbs. 2009;1(5):481-90.
57. Bradshaw EM, Chibnik LB, Keenan BT, Ottoboni L, Raj T, Tang A, et al. CD33 Alzheimer's disease locus: altered monocyte function and amyloid biology. Nature neuroscience. 2013;16(7):848-50.
58. Griciuc A, Serrano-Pozo A, Parrado AR, Lesinski AN, Asselin CN, Mullin K, et al. Alzheimer's disease risk gene CD33 inhibits microglial uptake of amyloid beta. Neuron. 2013;78(4):631-43.
59. Malik M, Simpson JF, Parikh I, Wilfred BR, Fardo DW, Nelson PT, et al. CD33 Alzheimer's risk-altering polymorphism, CD33 expression, and exon 2 splicing. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2013;33(33):13320-5.
60. Huang KL, Marcara E, Pimenova AA, Di Narzo AF, Kapoor M, Jin SC, et al. A common haplotype lowers PU.1 expression in myeloid cells and delays onset of Alzheimer’s disease. Nature neuroscience. 2017.

61. Malik M, Parikh I, Vasquez JB, Smith C, Tai L, Bu G, et al. Genetics ignite focus on microglial inflammation in Alzheimer’s disease. Molecular neurodegeneration. 2015;10:52.

62. Efthymiou AG, Goate AM. Late onset Alzheimer’s disease genetics implicates microglial pathways in disease risk. Molecular neurodegeneration. 2017;12(1):43.

63. Hildebrandt MA, Hoffmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen YY, et al. High-fat diet determines the composition of the murine gut microbiome independently of obesity. Gastroenterology. 2009;137(5):1716-24 e1-2.

64. Turnbaugh PJ, Backhed F, Fulton L, Gordon JL. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell host & microbe. 2008;3(4):213-23.
Figure Legends

**Figure 1. Cladograms reveal microbial phylogenetic branches associated with APOE status.** Taxa are represented as nodes and are connected by lines based on the phylogenetic relatedness of all the taxa present in each experimental cohort. For example, the end node, a, in both the females (A) and males (B) represents the genus *Actinobacteria* and is connected to other nodes representing the higher level taxa related to *Actinobacteria* including b the family Bifidobacteriaceae, c the order Bifidobacteriales, and d the class Actinobacteria. Nodes are colored based on significance cutoffs for the statistically significant APOE status. Many groups are associated with APOE in males, with fewer in females. Highlighted nodes indicate statistical significance (p< 0.05 for Kruskal-Wallis/Wilcoxon tests and a logarithmic LDA score >2.0 threshold.

**Figure 2. Microbiome alpha and beta-diversity as a function of APOE.** Alpha diversity is depicted as boxplots (A-B) and beta diversity as PCoA plots (C-D). These results are from mice at three months of age. Statistical significance for the findings is indicated below each graph. Ellipses in C and D represent 95% confidence intervals. Females (C) $R^2 = 0.058$. Males (D) $R^2 = 0.146$.

**Figure 3. Heatmaps depict overall microbiota profiles grouped by APOE status.** These heatmaps depict per-sample relative abundance for family-level bacteria in female (A) and male (B) mice. Columns were grouped by APOE status, rows were grouped by the Ward clustering algorithm using the Pearson Correlation Coefficient distance measures.
Figure 4. The phylogenetic branch defined by Clostridia and its lower taxa shows a significant association with APOE in male mice. The relative abundance of each depicted bacteria, except for Lachnospiraceae, was significantly associated with APOE status. The relative abundance of these bacteria decreased in a stepwise fashion from APOE2 to APOE3 to APOE4. P values have been corrected using an FDR approach. These data are derived from the three-month male mice with data for all ages provided in Supplementary Tables S1.1-S3.2.

Figure 5. The phylogenetic branch defined by Erysipelotrichia and its lower taxa shows a significant association with APOE in male mice. All depicted bacteria were significantly associated with APOE status. The relative abundance of these bacteria increased in a stepwise fashion from APOE2 to APOE3 to APOE4. P values have been corrected using an FDR approach. These results are derived from three-month male mice.

Figure S1A-B. Cladograms for five- and seven-month female and male mice, respectively, reveal microbial phylogenetic branches associated with APOE status. These results depict taxa significant associated with APOE status by LefSe analysis.
Cladograms reveal microbial phylogenetic branches associated with APOE status. Taxa are represented as nodes and are connected by lines based on the phylogenetic relatedness of all the taxa present in each experimental cohort. For example, the end node, a, in both the females (A) and males (B) represents the...
genus Actinobacteria and is connected to other nodes representing the higher level taxa related to Actinobacteria including b the family Bifidobacteriaceae, c the order Bifidobacteriales, and d the class Actinobacteria. Nodes are colored based on significance cutoffs for the statistically significant APOE status. Many groups are associated with APOE in males, with fewer in females. Highlighted nodes indicate statistical significance (p< 0.05 for Kruskal-Wallis/Wilcoxon tests and a logarithmic LDA score >2.0 threshold.

Figure 2
Microbiome alpha and beta-diversity as a function of APOE. Alpha diversity is depicted as boxplots (A-B) and beta diversity as PCoA plots (C-D). These results are from mice at three months of age. Statistical significance for the findings is indicated below each graph. Ellipses in C and D represent 95% confidence intervals. Females (C) R² = 0.058. Males (D) R² = 0.146.

**Figure 3**

Heatmaps depict overall microbiota profiles grouped by APOE status. These heatmaps depict per-sample relative abundance for family-level bacteria in female (A) and male (B) mice. Columns were grouped by APOE status, rows were grouped by the Ward clustering algorithm using the Pearson Correlation Coefficient distance measures.
Figure 4

The phylogenetic branch defined by Clostridia and its lower taxa shows a significant association with APOE in male mice. The relative abundance of each depicted bacteria, except for Lachnospiraceae, was significantly associated with APOE status. The relative abundance of these bacteria decreased in a stepwise fashion from APOE2 to APOE3 to APOE4. P values have been corrected using an FDR approach. These data are derived from the three-month male mice with data for all ages provided in Supplementary Tables S1.1-S3.2.
The phylogenetic branch defined by Erysipelotrichia and its lower taxa shows a significant association with APOE in male mice. All depicted bacteria were significantly associated with APOE status. The relative abundance of these bacteria increased in a stepwise fashion from APOE2 to APOE3 to APOE4. P values have been corrected using an FDR approach. These results are derived from three-month male mice.
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1A.Cladograms5memalesandfemales.pdf
- ZajacetalAP0EMicrobiome2021SupplementalTables.xlsx
- FigureS1B.Cladograms7memalesandfemales.pdf